Strategies to increase -cell mass expansion

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Strategies to increase β-cell mass expansion

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Table of contents

Abstract

Aim: Failure of the functional β-cell mass to adapt to compensate for peripheral insulin resistance leads to the development of type 2 diabetes and gestational diabetes. The overall aim of this thesis was to study mechanisms regulating β-cell mass expansion, using pregnancy in mice as an experimental model in which the β-cell mass increases during gestation and returns to normal levels post-partum. The mechanisms underlying this adaptation are not well understood, although placental signals are thought to be involved. The first objective of the project was to analyse changes in the β-cell mass during pregnancy, and post-partum. The second objective was to quantify the expression of islet β-cell G protein-coupled receptors (GPCRs) and their placental ligands to identify novel placental signals potentially involved in β-cell adaptation to pregnancy. The third objective was to examine the effects of one of the novel placental signals, R-spondin 4 (RSPO4), on β-cell function.

Methods: β-cell proliferation was quantified using immunohistochemical staining of pancreatic sections labelled with 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue. β-cell identity was confirmed by co-immunostaining for insulin and quantified by morphometric analysis. Islet GPCR and placental ligand mRNAs were quantified using a non-biased quantitative real time PCR (qRT-PCR) array approach. The effects of RSPO4 on insulin secretion, β-cell proliferation and survival were evaluated using radioimmunoassay, BrdU incorporation and caspase 3/7 apoptosis assays, respectively.

Results: Mouse β-cells proliferated during pregnancy, peaking around gestational day 12, are the newly-formed β-cells which were not selectively lost post-partum. Placental expression of GPCR ligands was upregulated on day 12, and islet GPCR expression was differentially regulated during pregnancy. One candidate placental GPCR ligand, RSPO4, had pro-proliferative and insulinotropic effects in β-cells, consistent with an adaptive function during pregnancy.

Conclusion: The placenta synthesizes many GPCR ligands, such as RSPO4, which have the potential to influence β-cell function during pregnancy, and which may have therapeutic potential in treating diabetes.

List of abbreviations

AKAP	A kinase anchoring protein
AKT (PKB)	Protein kinase B
AP-2	β2-adaptins
ATP	Adenosine triphosphate
Bcl6	B-cell lymphoma 6 protein
BMI	Body mass index
BrdU	5-bromo-2'-deoxyuridine
cAMP	Cyclic adenosine monophosphate
CaSR	Calcium sensing receptor
CCL11	Eotaxin
CCL21	Chemokine (C-C motif) ligand 21
CCL7	Chemotactic protein-3
CDK1	Cyclin-dependent kinase 1
CDK4	Cyclin-dependent kinase 4
CHOP	CCAAT-enhancer-binding homologous protein
C-MET	Hepatocyte growth factor receptor
C-MYC	V-Myc avian myelocytomatosis viral oncogene homolog
CNS	Central nervous system
CPT-1	Carnitine palmitoyltransferase 1
CX3CL1	Fractalkine
DAG	Diacylglycerol
EDTA	Ethylenediaminetetraacetate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal–regulated kinase (MAPK)
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate

FoxO1	Forkhead box protein O1
GAD65	Glutamic acid decarboxylase
GAPs	GTPase-activating proteins
GDM	Gestational diabetes mellitus
GH	Growth hormone
GLP1	Glucagon-like peptide 1 receptor
GLUT	Glucose transporter
GPCR	G protein coupled receptor
GR	Glucocorticoid receptor
GSIS	Glucose stimulated insulin secretion
HbA1c	Glycated haemoglobin
HCC-1	Hemofiltrate CC chemokine-1
HCC-4	Hemofiltrate CC chemokine-4
HGF	Hepatocyte growth factor
hGH-N	Human pituitary growth hormone
hGH-V	Human placental growth hormone
HNF-4α	Hepatocyte nuclear factor-4α
hPL	Human placental lactogen
HTR1D	Serotonin receptor 1d (5-hydroksytryptamine receptor 1d)
HTR2B	Serotonin receptor 2b (5-hydroksytryptamine receptor 2b)
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
IL-1β	Interleukin 1β
INF-γ	Interferon γ
IPGTT	Intraperitoneal glucose tolerance test
IP3	Inositol 1,4,5-trisphosphate
IRS	Insulin receptor substrate

Jak2	Janus kinase 2
LTB4	Leukotriene B4
LTB4R1	Leukotriene B4 receptor 1
MafA	V-Maf masculoaponeurotic fibrosarcoma oncogene homologue A
MAPK	Mitogen activated protein kinase
MDC	Macrophage-derived chemokine
MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein-1β
MKP-1	Dual-specificity phosphoprotein phosphatase 1
NeuroD1	Neurogenic differentiation 1
NHERF2	Na+/H+ exchanger regulatory factor 2
NOD mice	Non-obese diabetic mice (type 1 diabetes mellitus model)
p18	Cyclin-dependend kinase 4 inhibitor C (CDKN2C)
p21	Cyclin-dependent kinase inhibitor 1
p27	Cyclin-dependent kinase inhibitor 1B
p57	Cyclin-dependent kinase inhibitor 1C
Pdx-1	Pancreatic and duodenal homebox
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1-α
PA	Phosphatidic acid
PDZ	Post-synaptic density of 95 kDa (PSD95)-disc large-zona occludens (PDZ)
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinozytol 3-kinase
PIBF	Progesterone induced blocking factor 1
PKA	Protein kinase A
PKC	Protein kinase C

PL	Placental lactogen
PLD	Phospholipase D
PPARα	Peroxisome proliferator-activated receptor α
PRL	Prolactin
PRLR	Prolactin receptor
PTH1R	Parathyroid hormone 1 receptor
PTH2R	Parathyroid hormone 2 receptor
Rasd1	Dexamethasone-induced ras-related protein 1
RSPO4	R-spondin 4
SAT	Subcutaneous adipose tissue
SEM	Standard error of the means
SERCA2	Sarcoendoplasmic reticulum Ca2+-ATPase 2
SHC	SH2-plekstrin homology domain
STAT3	Signal transducer and activator of transcription 3
STAT5	Signal transducer and activator 5
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
T3DM	Type 3 diabetes mellitus
TGF-β1	Transforming growth factor β
TNF-α	Tumour necrosis factor α
TPH1/2	Tryptophan hydroxylase 1 and 2
TRB3	Tribble 3
Tris	Tris(hydroxymethyl)aminomethane
ucCO	Under-carboxylated osteocalcin
UCP-2	Uncoupling protein 2
VAT	Visceral adipose tissue
VPAC1R	Vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide receptor
ZNT8	Zinc transporter 8

List of figures

List of tables

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Chapter 1

Introduction

Chapter 1

1.1 β-cells and diabetes

1.1.1 Islets of Langerhans

Islets of Langerhans, first identified in 1869, are the endocrine compartment of the pancreas and consist of four main types of endocrine cells: ~60% β-cells (secreting insulin), 20-30% α-cells (secreting glucagon), ~10% δ-cells (secreting somatostatin), <5% PP cells (expressing pancreatic polypeptide) and ~1% ε-cells (expressing ghrelin) [1, 2]. Rodent islets have a central β-cell core surrounded by a mantle of other endocrine cells. In contrast, there are no anatomical subdivisions in human islets, in which β-cells are intermingled with other cell types within the islet [3]. In addition, all of the islet cell types have equal access to blood vessels within the human islet, and the endocrine cells are not organized in layers around blood vessels. These observations are consistent with functional segregation and a lack of synchronized oscillatory activity of human islets as opposed to mice [3]. Only individual β-cells and small regions of human islets show synchronizes oscillations of β-cell intracellular Ca^{2+} levels, that become neutralized by the out-of-synch activity from other islet regions [3]. Islet α-, β-, δ-, ε- and PP cells are involved in maintaining energy balance and regulation of glucose metabolism through interconnected functions [4].

1.1.2 Insulin secretion and action

Insulin is secreted by pancreatic β-cells and plays the key role in the regulation of glucose metabolism. The single human insulin gene, 1355 base pairs long, is located on chromosome 11, while rodents possess two copies of the insulin gene [5]. Plasma glucose concentrations regulate the synthesis of insulin at transcriptional and post-transcriptional levels [6]. β-cell specific transcription factors Pdx-1 (Pancreatic and duodenal homebox), NeuroD1 (Neurogenic Differentiation 1) and MafA (V-Maf masculoaponeurotic fibrosarcoma oncogene homologue A) are crucial for regulation of insulin gene transcription by glucose [6].

Insulin is expressed initially as preproinsulin (11.5 kDa) which is processed in the rough endoplasmic reticulum to a 9 kDa proinsulin precursor molecule. Proinsulin consists of chains A and B joined by C peptide. Proinsulin is packaged into secretory granules in the Golgi complex where it is being processed by endopeptidases (prohormone convertases 2 and 3) and carboxypeptidase H to insulin (5.8 kDa). Processing of proinsulin into insulin continues in the secretory granules. Under normal physiological conditions, secretory granules contain more than 95% of insulin and less than 5% proinsulin [1].

Insulin secretion is regulated by circulating nutrients as well as hormones and neural factors [7-9]. The main stimulus of insulin secretion is glucose stimulated insulin secretion (GSIS). Glucose is transported into β-cells through glucose transporters (GLUT1, 2 and 3 in humans, GLUT2 in rodents) followed by glucose phosphorylation by glucokinase. Formation of glucose-6-phosphate is the initial step of glycolysis. Glucose metabolism leads to an increased ATP concentration, closure of ATP-sensitive K^+ (K_{ATP}) channels, depolarization of the β-cell membrane, opening of the voltage-dependent Ca^{2+} channels and increased influx of Ca^{2+}. The resultant increase in intracellular Ca^{2+} initiates insulin secretion [10]. Glucose-induced insulin secretion is biphasic, with a rapid but short-lived first phase (5-10 min) followed by a slowly developing sustained second phase. The second phase of insulin secretion from isolated mouse islets is characterized by oscillations which have a period of 10-30 s [11]. On the other hand, insulin secretion from the perfused human pancreas is characterized by oscillations with a 5-10 min period [12]. Type 2 diabetes mellitus (T2DM) is characterized by the loss of first-phase secretion and reduced second-phase secretion. In addition, decreased first-phase GSIS is a symptom of the early stage T2DM and impaired glucose tolerance [10].

The main target tissues for insulin are skeletal muscle, adipose tissue and liver. In the liver insulin promotes uptake and storage of carbohydrates, amino acids and fat, while at the same time reducing catabolic processes. Moreover, it inhibits glycogenolysis and gluconeogenesis while increasing glycogenogenesis in the liver. The main effects of insulin in the skeletal muscle are to stimulate glucose

transport and glucose storage in the form of glycogen as well as stimulation of glycolysis and the activity of tricarboxylic acid cycle. In adipose tissue, insulin stimulates glucose uptake, glycerol synthesis and triglyceride formation at the same time as blocking lipolysis [1]. Furthermore, insulin has anti-apoptotic and pro-proliferative properties in β-cells [13]. Insulin binds to the insulin receptor (IR) in target tissues and exerts its functions through a signalling cascade involving insulin receptor substrate proteins (IRS), phosphatidylinositol 3 (PI3)-kinase, AKT/protein kinase B (AKT/PKB) and mitogen activated protein kinase (MAPK) [1].

1.1.3 Diabetes

Type 1 diabetes mellitus (T1DM) is an autoimmune disease affecting 10% of adults suffering from diabetes and it is one of the most common chronic diseases of children [14]. In T1DM autoreactive lymphocytes kill β-cells as a result of defects in central and peripheral B and T cell tolerance [15]. The development of diabetes is associated with the presence of autoantibodies in more than 90% of patients with T1DM. In animal models, insulin has been identified as the primary initiating antigen whereas other autoantigens (e.g. glutamic acid decarboxylase (GAD65) and zinc transporter 8 (ZNT8)) have been associated with progression of the disease in humans [14]. There is a strong genetic factor involved in the pathogenesis of T1DM, mainly alleles within the major histocompatibility complex (MHC), which is manifested by the four-fold higher risk of developing islet auto-antibodies by children who have a family history of T1DM compared to children with no family history [16]. However, 90% of patients with new onset disease do not have relatives with T1DM which suggests the importance of environmental factors leading to T1DM [14].

T2DM is the most common type of diabetes, affecting 90% of diabetic patients. Genetically and epigenetically susceptible people develop T2DM when they fail to adapt to energy surplus [17]. Metabolic symptoms of T2DM include: a loss of β-cell ability to compensate for the fuel surfeit, peripheral insulin resistance, increased production of endogenous glucose, increased glucagon secretion, reduced incretin response, hypoadiponectinemia, inflammation of adipose tissue,

and limited expansion of subcutaneous adipose tissue (SAT) [17]. Glucotoxic and glucolipotoxic mechanisms accelerate β-cell failure and the reduction in β-cell mass in patients suffering from T2DM. Many obese people do not develop T2DM because of partitioning of excess calories in SAT, which is related to increased β-cell mass, development of minimal insulin resistance and increased expansion of SAT in relation to visceral adipose tissue (VAT) [17]. Insulin resistance is one of the main causes of T2DM and is characterized by hyperinsulinemia and hyperglycemia in fasting conditions, increased glycated haemoglobin (HbA1c), hyperlipidemia, impaired glucose and insulin tolerance, increased glucose production in the liver and loss of first phase insulin secretion [18]. It is believed that energy surfeit sensing is mediated by ATP and adenosine monophosphate-activated protein kinase (AMPK) [18].

Insulin resistance and insulin deficiency within the central nervous system (CNS) are referred to as type 3 diabetes mellitus (T3DM) [19]. It is believed Alzheimer's disease (AD) represents T3DM. Initial cognitive impairments in Alzheimer's disease are accompanied by dysfunctional glucose metabolism and energy balance in the brain [20]. Therefore, it has been proposed that T3DM is a major pathogenic factor in AD.

Any degree of glucose intolerance with onset during pregnancy is classified as gestational diabetes mellitus (GDM) [21]. During pregnancy, the mother's β-cell mass increases to compensate for the increased metabolic demand and impairment of those adaptive mechanisms can lead to GDM. GDM is a result of pre-existing insulin resistance or impaired β-cell expansion during pregnancy. The main independent risk factors for GDM are obesity as well as high energy and saturated fat diet [22, 23]. The weight gain of 2.3 to 10 kg/year in the 5 years preceding pregnancy elevates the risk of GDM [22]. In addition, mothers with low own birth weight are more likely to develop gestational diabetes [24]. GDM increases the risk of perinatal complications and developing T2DM in women and offspring postpartum [25-27]. A dysfunction in maternal β-cells leads to GDM and T2DM post-partum. Furthermore, a six-year study of the offspring of mothers with pregestational diabetes (T1DM and T2DM) and GDM revealed that maternal

diabetes is linked to obesity as well as poorer intellectual and psychomotor development of the offspring [27].

1.2 Hormonal control of β-cell adaptation to pregnancy

During pregnancy the mother undergoes major hormonal and metabolic changes to ensure a continuous supply of nutrients to the fetus (Figure 1.1). Adaptations to increased body weight and insulin demand in pregnant mice involve increased islet β-cell proliferation and islet volume, insulin synthesis, glucose-stimulated insulin secretion, glucose metabolism as well as upregulated expression of glucokinase and glucose transporters (GLUT2 and GLUT5) [28-33]. The mother develops insulin resistance during normal pregnancy to ensure a continuous supply of nutrients to the fetus. Impaired maternal β-cell adaptation leads to GDM and increases the risk of developing post-partum diabetes in rats and women [34, 35]. The slow basal rate of adult β-cell proliferation is accelerated in response to an increased insulin demand resulting from pregnancy, obesity or insulin resistance [34, 36, 37]. During rodent pregnancy, the β-cell mass increases by 2.6-fold [38]. The increase in the β-cell mass in human pregnancy is smaller than in rodents, a 1.4-fold increase in the pancreatic fractional area in the first trimester of pregnancy [39]. Furthermore, an increase in the human β-cell mass during pregnancy is mainly caused by an increased number of β-cells in new, small islets as opposed to an increase in the islet size due to a higher replication rate observed in rodent pregnancy [39]. There are limited data on the human β-cell mass adaptations during pregnancy but it has been shown that β-cell replication is not significantly changed during pregnancy [39]. In rodents, β-cell proliferation is maximal at gestational day 13-15, dropping to control values at day 18-19 [29, 34]. Despite the apparent differences between rodent and human adaptation to pregnancy, placental lactogen and prolactin (PRL) play a main role in β-cell upregulation during pregnancy and are central to the pregnancy adaptation mechanism in those species [30].

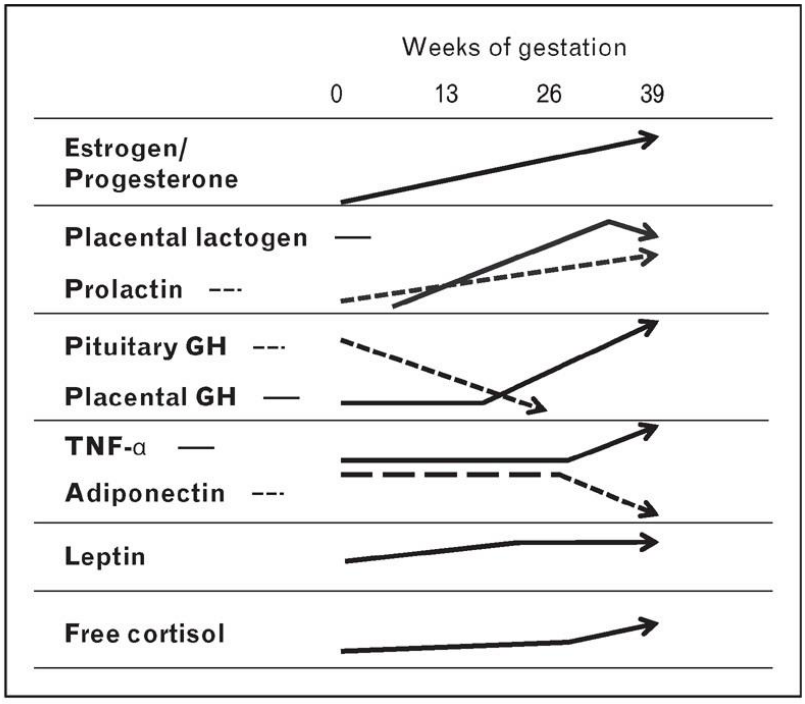

Figure 1.1. Major hormonal changes in pregnant women during pregnancy. Estrogen (oestrogen) is involved in development of placenta and increases prolactin production. Progesterone stimulates maternal food intake and prevents rejection of the fetus by suppression of the maternal immune system. A rise in maternal circulating cortisol, growth hormone (GH), progesterone, tumour necrosis factor α (TNF-α) and a drop in plasma adiponectin are involved in decreasing maternal insulin sensitivity [40].

1.2.1 Lactogenic hormones

Lactogenic hormones, including PRL and placental lactogens, stimulate the development of lactocytes in the mammary gland and lactation after parturition [41]. Lactogenic hormones affect the β-cell mass both in the mother and in the fetus during pregnancy. A rise in the levels of maternal serum PRL and placental lactogen in rodents is related to a 2-fold increase in the mother's islet mass [29]. Moreover, in pregnant women, levels of PRL and placental lactogen (PL) are increased which parallels expansion of the β-cell mass [40]. The rapid rate of β-cell replication observed in the fetus during gestation and infancy is crucial for determining the final adult β-cell mass [37, 42]. It has been also suggested that nutritional, genetic and environmental factors interfering with β-cell replication at this stage can influence the adult β-cell mass and the risk of developing diabetes in adults [37].

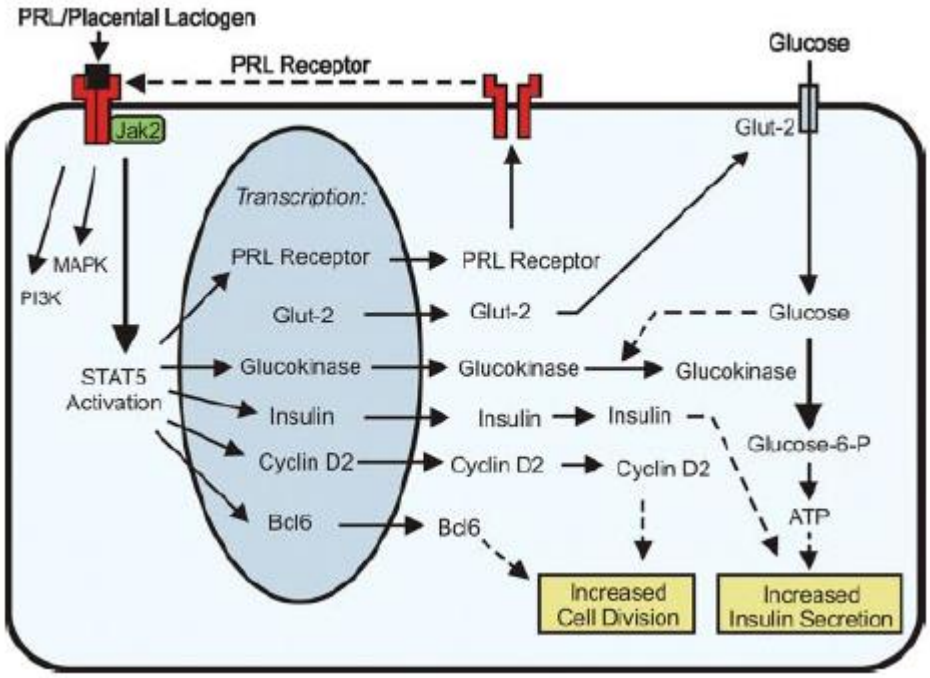

Figure 1.2. Schematic representation of mechanisms involved in the lactogenic hormone-controlled regulation of β-cell mass in rodents. PRLR activation plays a main role in β-cell adaptation to pregnancy, leading to an increase in insulin secretion and β-cell proliferation. Glut-2 - glucose transporter 2, Jak2 - Janus kinase 2, PI3K - phosphatidylinozytol 3-kinase [43].

Furthermore, in rats, PRL facilitates glucose uptake and utilization through increased expression of GLUT2 and glucokinase as well as upregulated activity of pyruvate dehydrogenase and enhanced GSIS (Figure 1.2) [44, 45].

Interestingly, glucose increases expression of rat islet prolactin receptor (PRLR) and potentiates the stimulatory effect of PRL on cell cycle gene expression (cyclins A2, B1, B2, D2 E1 and cyclin dependent kinase 1 (CDK1)) [45].

The importance of PRL in the process of maternal adaptation to insulin resistance is suggested by results of *in vivo* and *in vitro* experiments. For example, PRL and human PL (hPL) stimulate β-cell replication, GSIS and prolong β-cell survival in isolated human and rodent islets [40]. Overexpression of hPL in primary β-cells and insulionoma cells increases β-cell replication and extends β-cell lifespan [40]. A global knockout of PRLR in mice reduces the β-cell mass by 26-42%, as

compared to wild type littermates, and blunts GSIS responses [46]. Furthermore, islets of pregnant global heterozygous PRLR knockout mice are characterized by a changed expression pattern of various genes, including Janus kinase 2 (Jak2), AKT, menin and cell cycle suppressors p18 (cyclin-dependent kinase 4 Inhibitor C) and p21 (cyclin-dependent kinase inhibitor 1) as compared to wild type mice [47]. Mutations in the menin (multiple endocrine neoplasia type 1) gene are linked to a predisposition to hyperplasia of the parathyroid glands and pituitary as well as tumours of the endocrine pancreas [48]. Overexpression of menin in mice prevents β-cell mass expansion during pregnancy [49]. Moreover, during pregnancy in PRLR wild type mice expression of phospho-Jak2, IRS-2, phospho-AKT, AKT and p21 is increased whereas menin and p18 expression is downregulated [47].

In the proposed mechanism of PRL action in mouse pregnancy, PRL increases expression of AKT leading to a rise in p21 expression. An elevated level of p21 may prevent uncontrolled cell proliferation and stabilize cyclin D-CDK4 (cyclin-dependent kinase 4) complex, ensuring regulated progression through cell cycle (Figure 1.2) [47]. In addition, downregulation of the menin/p18 complex and increased activation of Jak2 may be responsible for enhanced β-cell proliferation. These responses are blocked in pregnant PRLR heterozygous mice [47].

Within islets only β-cells express PRLR [43]. Binding of PRL to its receptor activates Jak2, which phosphorylates signal transducer and activator 5 (STAT5), leading to its translocation to the nucleus, where it activates expression of target genes (Figure1.2) [50]. It has also been shown that STAT5 action may be mediated by Bcl6 (B-cell lymphoma 6 protein) transcription factor [43]. Activation of STAT5 by PRL is biphasic, with peaks within 30 min and between 1 and 3 hours followed by prolonged activation after 4 hours [51]. Moreover, tonic stimulation of islets by either PRL or GH is required for responsiveness of islets to glucose in normal conditions in rats [50]. In addition, experimental data indicate that PL activates cyclin D2 expression, which is mediated by STAT5 [52]. A recent study on rat INS-1 β-cells has demonstrated that STAT5B overexpression mimics the

negative effects of PRL on expression of FoxO1 (Forkhead box protein O1), p27 (cyclin-dependent kinase Inhibitor 1B), p57 (cyclin-dependent kinase inhibitor 1C) and menin [45]. PRL also activates insulin receptor substrates/phosphatidylinositol 3-kinase (IRSs/PI3K) and the MAPK pathway in islets of neonatal and pregnant rats [53].

The peak of β-cell proliferation on gestational day 14 in pregnant mice coincidences with strong upregulation of β-cell serotonin-synthesizing enzymes, tryptophan hydroxylase 1 and 2 (TPH1/2) [54]. Brain knockdown of serotonin receptor 2C (HTR2C) in non-pregnant mice exacerbates symptoms of T2DM [55]. Experiments on mice involving serotonin agonists and antagonists have shown that lactogenic hormones induce TPH1/2 expression and serotonin synthesis in islets, which is consistent with a model where serotonin stimulates β-cell proliferation through its receptor, Gαq-coupled HTR2B [56, 57]. Just before parturition, β-cell proliferation and the β-cell mass decrease, returning to the pre-pregnancy levels. This decrease is related to decreased expression of HTR2B and increased expression of Gαi-coupled HTR1D [56]. Serotonin also increases GSIS in pregnant mice through activation of HTR3 receptor, a cation-selective ion channel, and lowering the glucose threshold for insulin secretion [58]. Another function of serotonin is mood regulation, acting via CNS, which may explain the association between depression and diabetes (T2DM and GDM) [56]. Serotonin synthesised in β-cells plays an important role in mediating the effects of lactogenic hormones on islets.

1.2.2 Hepatocyte Growth Factor signalling

In experiments performed on pregnant rats Johansson and colleagues showed that proliferation of islet endothelial cells precedes β-cell proliferation [59]. In addition, endothelial cells can stimulate β-cell proliferation *in vitro* through secretion of hepatocyte growth factor (HGF). HGF expression is enhanced on gestational day 15, which correlates with maximum β-cell proliferation [59]. Knockout of the pancreatic HGF receptor (C-MET) in rats diminishes maternal β-

cell proliferation and increases β-cell apoptosis on gestational day 15, resulting in reduced β-cell expansion, glucose intolerance, increased blood glucose and decreased plasma insulin, symptoms which are hallmarks of GDM [60], These C-MET knockout rats are also characterized by reduced expression levels of islet PRLR, STAT5 and Forehead box M1 as well as upregulation of p27 mRNA. HGF may regulate gene expression through hepatocyte nuclear Factor-4α (HNF-4α) and ERK. Both HGF and HNF-4α activate ERK signalling cascade in β-cells [59]. In addition, HNF-4α is necessary for expansion of the β-cell mass in response to increased metabolic demand in pregnancy [61]. It is not yet clear whether HGF regulates β-cell expansion during pregnancy directly or indirectly through PRL/PL signalling.

1.2.3 Glucocorticoids

A stimulatory effect of lactogenic hormones on β-cell proliferation is counteracted by increased plasma levels of glucocorticoids post-partum in rats [62]. Three days after delivery of pups a burst of apoptosis and a drop in proliferation of maternal β-cells is observed in rats, which correlates with DNA fragmentation and decreased phosphorylation of AKT, an anti-apoptotic kinase [63]. In addition, it has been shown that endoplasmic reticulum (ER) stress and unfolded protein response are involved in this process. In this mechanism C/EBP homologous protein (CHOP) increases expression of Tribble 3 (TRB3) which in turn inhibits AKT leading to increased β-cell apoptosis post-partum [63].

Furthermore, β-cell renewal and function in late pregnancy and early lactation are modulated by STAT5B/glucocorticoid receptor (GR) complex [64]. Elevated levels of glucocorticoids post-partum trigger formation of a STAT5B/GR complex, which then activates transcription of dexamethasone-induced Ras-related protein 1 (Rasd1) in pancreatic β-cells. Upregulation of Rasd1 causes reduced insulin secretion through inhibiting protein kinase A (PKA), protein kinase C (PKC) and ERK. PRL has the opposite to glucocorticoids effect on Rasd1 expression. Activation of Rasd1 expression by glucocorticoids represents a mechanism for reducing β-cell function during early lactation [64]. In addition, glucocorticoids are

responsible for the post-partum decrease in expression of sarcoendoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), leading to decreased insulin secretion during lactation. This effect depends on downregulation of signal transducer and activator of transcription 3 (STAT3) [65]. Furthermore, glucocorticoid-mediated dephosphorylation of phosho-ERK leads to reduced β-cell proliferation in lactating rats [66]. Dual-specificity phosphatase 1 (MKP1), downstream of glucocorticoids, is involved in this process [66].

Lactogens and glucocorticoids have also opposing effects on gene expression and differentially regulate fatty acid oxidation and GSIS in β-cells [44]. In rat insulinoma cells, PRL decreases expression of FoxO1 and genes involved in biogenesis of mitochondria and peroxisomes, including peroxisome proliferator-activated receptor γ coactivator 1-α (PGC-1α) and peroxisome proliferator-activated receptor α (PPARα) transcription factors whereas dexamethasone (DEX) and hydrocortisone stimulate expression of those proteins and uncoupling protein 2 (UCP-2) [60]. FoxO1 induces apoptosis and inhibits proliferation in pancreatic β-cells [44]. Down-regulation of FoxO1 by prolactin is mediated by STAT5. It has been proposed that PRL-mediated inhibition of FoxO1 expression constitutes a new mechanism for promoting β-cell proliferation and increased β-cell mass in late gestation, which counteract effects of the increased cortisol concentration at this stage of pregnancy [44].

1.2.4 Oestradiol

Oestradiol may regulate β-cell expansion during pregnancy and in obese rats through repression of microRNA-338-3p [67]. On day 14 of gestation in rats, the peak of the β-cell mass expansion, increased expression of microRNA-144 and microRNA-451 is observed. At the same time, expression of microRNA-218 and micro-RNA-338-3p is downregulated. Deactivation of micro-RNA-338-3p in β-cells using specific anti-microRNA promotes proliferation and protects β-cells from apoptosis whereas its upregulation mimics the reduction of β-cells at the end of pregnancy [67]. Overexpression of microRNA-338-3p in rat islet cells prevents proliferation induced by oestradiol. In addition, in isolated rat islets, activation of

the G protein-coupled oestradiol receptor (GPR30) and the glucagon-like peptide 1 (GLP-1) receptor leads to a decrease in the level of micro-RNA-338-3p [67]. The effect of oestradiol is cAMP-dependent [67]. Furthermore, maximum β-cell expansion in pregnancy correlates with increased expression of GPR30. Experimental data also suggests that in non-pregnant rats, incretins may control the level of microRNA-338-3p [67].

Maternal adaptations to pregnancy involve changes in plasma levels of a wide range of hormones. Lactogenic hormones play a central role in β-cell mass expansion and insulin secretion upregulation during pregnancy but other important factors, oestradiol and HGF, are also involved. Moreover, the regulatory mechanisms include glucocorticoids, which are responsible for the reversal of β-cell expansion post-partum. Dysregulation of the β-cell mass expansion control mechanisms may result in GDM, leading to perinatal complications and development of T2DM in the mother and offspring post-partum.

1.3 Placenta functions

The placenta induces changes in the maternal blood flow, appetite and energy homeostasis in order to provide an ample supply of nutrients for itself and the fetus. Glucose is the main source of energy for the placenta and the fetus, crossing into the placenta from maternal circulation by facilitated transport. Human placental hormones, placental lactogens and placental growth hormone, enhance food intake in order to increase maternal blood glucose and maximise its transfer to the fetus [68]. They augment food intake during early pregnancy while triggering utilization of those reserves to support fetal growth and lactation at term [40, 69]. Located on chromosome 17, human pituitary growth hormone (hGH-N, GH1), placental growth hormone (hGH-V, GH2) and placental lactogens (hPL-A, hPL-B, hPL-L) genes have high homology of amino acid sequences. The placenta secretes all of those hormones except hGH-N.

By the end of the first trimester, progesterone and hPLs drive an increase in food intake and appetite, which, combined with a low metabolic demand of the fetus at

that stage, leads to increased deposition of fat [70]. Leptin, secreted by adipocytes, normally suppresses food intake signals in the hypothalamus. Consequently, dysregulation of energy balance mechanisms results in central leptin resistance [70]. In addition, the placenta secretes large quantities of leptin during pregnancy, which contributes to leptin resistance [71]. Results of experiments on rats suggest that this leptin resistance may cause central insulin resistance. Prolactin and placental lactogens may mediate the hormone-induced loss of response to leptin during pregnancy, acting on the hypothalamus [71]. Moreover, these hormones are known to stimulate β-cell mass expansion and insulin secretion, which facilitate fat deposition [40].

Insulin resistance, increased lipolysis as well as augmentation of maternal plasma glucose levels, circulating triglycerides and free fatty acids during pregnancy ensure continuous supply of nutrients to the fetus. Placental GH plays a main role in insulin resistance. Transgenic mice, overexpressing human placental GH, show symptoms of insulin resistance as manifested by fasting and postprandial hyperinsulinemia and minimal glucose lowering in response to insulin injection [72]. In the skeletal muscle of those mice, insulin-stimulated GLUT-4 translocation to the plasma membrane and insulin receptor expression are reduced compared to the wild type controls. Furthermore, overexpression of placental GH in mice results in increased expression of the regulatory p85 monomer of PI3-kinase, which negatively competes with the p85-p100 heterodimer of the kinase for binding to IRS-1. This competition is manifested by a reduced ability of insulin to stimulate IRS-1-associated activity of PI3-kinase [72].

1.4 The role of chemokines during pregnancy

Chemokines, also known as chemotactic cytokines, are small (8-15 kDa) signaling proteins, which are able to induce chemotaxis in responsive cells [73]. In addition to their immune system functions, those proteins are also involved in neoangiogenesis and tumour growth [74]. The chemokine protein family is comprised of more than 50 proteins. They are divided into four subfamilies (CXC, CC, C and CX3C) based on the occurrence and location of four distinct cysteine

residues that form intramolecular disulfide bonds [73]. Chemokines exert their functions through 20 G protein-coupled receptors (GPCRs), which has been identified to date.

Chemokines are a part of a large cytokine protein family. Cytokines are glycoproteins taking part in mammalian reproduction physiology, decidualization, trophoblast differentiation, implantation, embryogenesis, maternal recognition of pregnancy, placental growth and function, as well as term labour, delivery and lactation [75, 76]. Notably, maternal tolerance of the semi-allogenic fetus is achieved through an increase in the ratio of anti-inflammatory (e.g. IL-4, IL-10) cytokines, secreted by T-helper type 2 (Th2) lymphocytes, and pro-inflammatory (e.g. IL-12) cytokines, secreted by Th1 cells, which is triggered by progesterone-induced blocking factor 1 (PIBF) [77-79]. Furthermore, CCL2 and CCL5, secreted by decidua, attract dendritic cells (DCs) to the feto-maternal interface where they induce an increase in the ratio of the Th2/Th1 cells [80, 81]. Consequently, dysregulation of inflammatory mechanisms during pregnancy is implicated in aberrant placentation, preeclampsia and preterm labour [82-84].

In early pregnancy, macrophages and uterine-specific natural killer cells infiltrate the implantation site where they are believed to be important modulators of trophoblast invasion and decidualization [85]. mRNA of macrophage and natural killer chemoattractants is upregulated in mouse endometrium during endometrial receptivity and early pregnancy. The upregulated cytokines include monocyte chemotactic protein-3 (CCL7), eotaxin (CCL11), fractalkine (CX3CL1), macrophage inflammatory protein-1β (MIP-1β), chemokine (C-C Motif) ligand 21 (CCL21), hemofiltrate CC chemokine-1 (HCC-1) and -4 (HCC-4) as well as macrophage-derived chemokine (MDC) [85]. At the same time, PIBF ensures success of gestation via IL-10 and INFγ-mediated inhibition of natural killer (NK) cell activity [77]. The main sources of chemokines are epithelial cells and, after decidualization, differentiated stromal cells [85, 86].

The importance of cytokine signaling during pregnancy is highlighted by the fact that inflammation-related cytokine exposure plays a role in altered brain

development and offspring obesity [87]. In addition, obese mothers are characterized by elevated plasma levels of inflammatory markers and an increased risk of pregnancy complications [87].

1.5 G protein-coupled receptors

G protein-coupled receptors comprise the largest and most diverse group of membrane proteins in eukaryotes. 329 orthologue pairs of non-olfactory GPCRs have been identified in human and mouse genomes, with 85% of average percentage similarity of the orthologue pairs [88]. The abundance of GPCRs in the mammalian genome is reflected in their importance as potential therapeutic targets. Drugs targeting GPCRs are the most profitable and the largest group of medicines developed in recent years [89]. GPCRs are involved in cellular signal transduction in response to hormones and neurotransmitters as well as visual, taste and olfactory stimuli. At the molecular level, seven transmembrane α-helical domains can be distinguished in their structure, with an extracellular N-terminus and cytosolic C-terminus. There are five classes of structurally distinct GPCRs: rhodopsin (class A), secretin (class B), glutamate (class C), adhesion and Frizzled/Taste2 [90, 91]. The rhodopsin family is the largest and most diverse of these families.

GPCRs can function as monomers, homodimers, heterodimers and higher oligomers [92]. For example, the neurotensin NTS1 receptor monomer (class A) activates G proteins more efficiently than its dimeric form [93]. Receptor oligomerization can affect the nascent receptor membrane trafficking, pharmacology and signaling. The functional $GABA_B$ receptor, activated by γ-aminobutyric acid (GABA), exists as a heterodimer of $GABA_BR1$ and $GABA_BR2$ subunits. The $GABA_BR1$ subunit can reach the plasma membrane only after forming a complex with the $GABA_BR2$ subunit in the endoplasmic reticulum [94]. In addition, a functional heterocomplex of the serotonin receptor 5-HT2A and the glutamate receptor mGLUT2 has been shown to trigger unique cellular responses in the brain cortex of patients suffering from psychosis [95].

1.5.1 GPCR signalling

Although GPCRs share many structural and functional features, individual receptors exert unique physiological functions. Signal transduction through these receptors is initiated by binding of an agonist to the extracellular ligand-binding pocket, leading to conformational changes that are propagated to the cytoplasmic part of the receptor, responsible for G protein binding [96]. Conformational changes of the receptor promote coupling with its cognate heterotrimeric G proteins. The receptor catalyzes an exchange of GTP for GDP on the Gα subunit and dissociation of the GTP-bound Gα from the Gβγ subunit heterodimer (Figure 1.3). Free Gα and Gβγ subunits modulate activity of effector enzymes, including adenylate cyclase and phospholipase C, as well as ion channels, to generate second messengers (e.g. cAMP, IP3, DAG, Ca^{2+}).

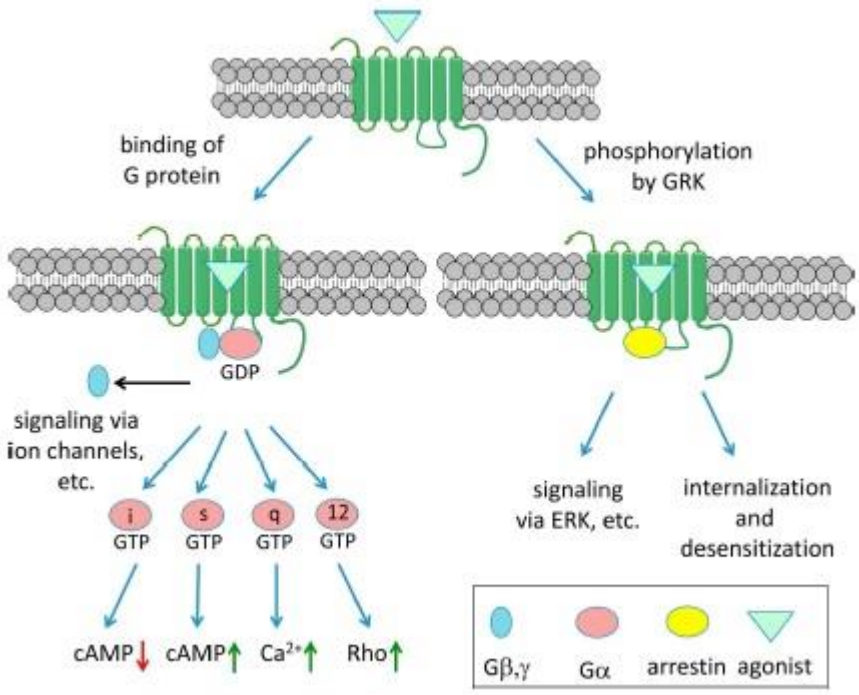

Figure 1.3. G protein-coupled receptor signalling. GPCR agonists can activate either the G protein-dependent or G protein-independent pathways, including the β-arrestin signaling pathway. GRK – GPCR kinase, ERK - extracellular signal–regulated kinase (MAPK) [89].

Subsequently, second messengers change activity of protein kinases (e.g. PKA, PKC) that regulate transcription factors and/or enzymes involved in the intermediary metabolism. Signaling is terminated by the intrinsic GTPase activity of the Gα subunit followed by a return of the G protein to its heterotrimeric state [89]. This is followed by receptor internalization from the cell surface to reduce the biological response, to resensitise the desensitised receptor by recycling or to propagate signals in the cytosol [97].

Within seconds of hormone binding, PKA/C kinases or G protein-coupled receptor kinases (GRKs) phosphorylate GPCRs. PKA/C-mediated phosphorylation leads to heterologous desensitization whereas GRK phosphorylation results in homologous desensitization (Figure 1.4). GRKs bind free Gβγ subunits, promoting subsequent β-arrestin binding to the receptor complex. β-arrestins recruit clathrin and β2-adaptins (AP-2), inducing agonist-receptor complex clustering in clathrin-coated pits and endocytosis dependent on dynamin (Dyn). The main role of β-arrestin is to uncouple the receptor from the G protein. An important role in the receptor sequestration plays GPCR activated phospholipase D (PLD), which hydrolyses phospholipids (e.g. phosphatidylcholine) to generate phosphatidic acid (PA). PA facilitates vesicle formation through initiation of membrane curvature changes [98]. Upon internalization GPCRs can be recycled back to the plasma membrane (resensitisation) or transported to lysosomes where they are being proteolysed (downregulation) [99].

One GPCR can signal through multiple signaling pathways and G proteins. For instance, the GLP-1 receptor can activate both Gαs and Gαq proteins, as well as cAMP-PKA and MAPK pathways [100].

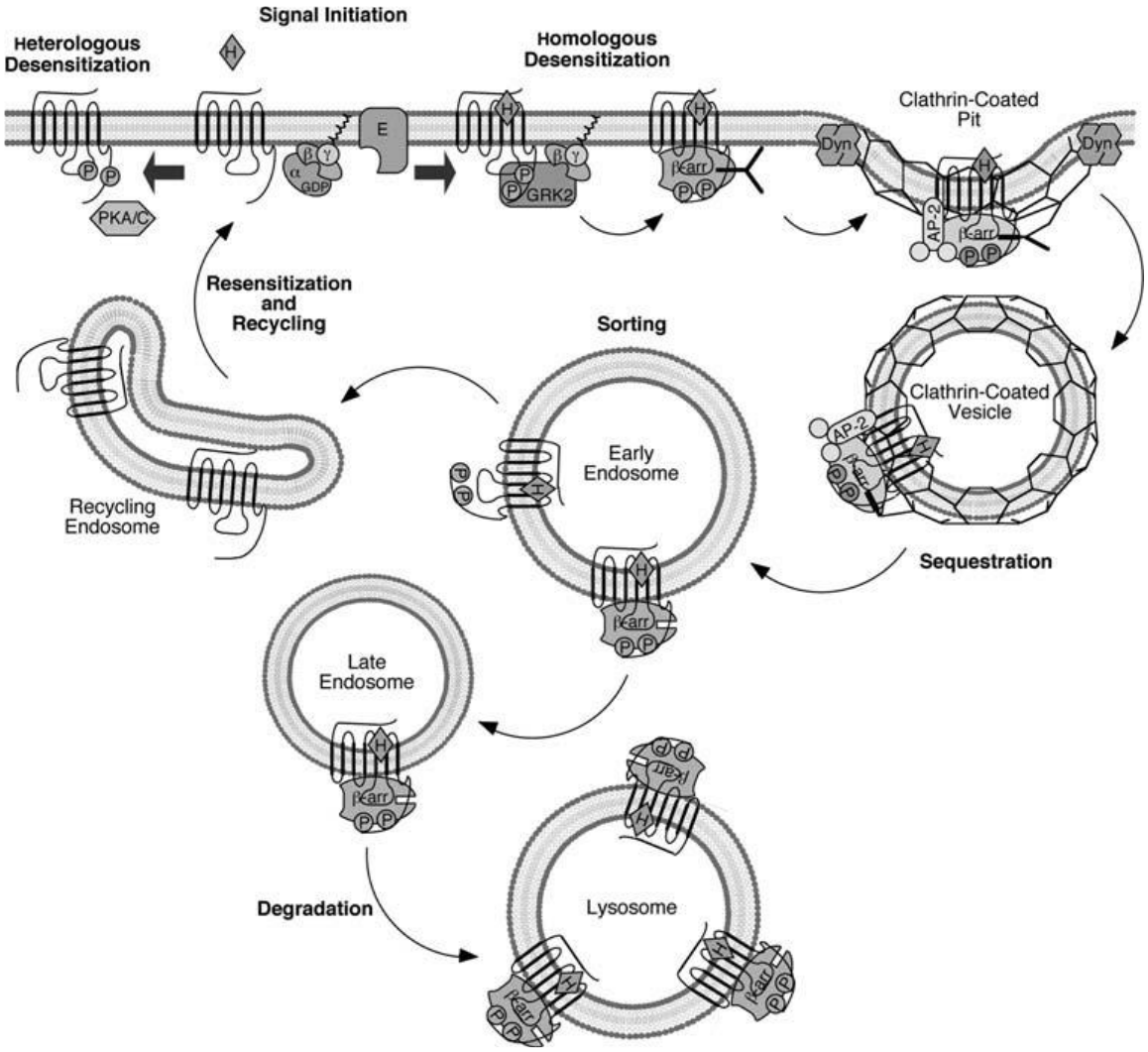

Figure 1.4. Desensitization, internalization and recycling of GPCRs. Phosphorylation of the activated receptor by GRK kinases results in the β-arrestin recruitment and subsequent internalisation of the receptor in clathrin-coated vesicles. The internalised receptor can be either targeted for degradation in lysosomes or recycled back to the plasma membrane. H – hormone; E – effector, ion channels or enzymes that generate second messengers; AP-2 – β2-adaptins; β-arr – β-arrestin; Dyn – dynamin; GRK2 – G protein-coupled receptor kinase 2 [91].

1.5.2 G proteins

G proteins are a group of heterotrimeric GTPases, consisting of a GTP-binding Gα subunit (39-52 kDa), which exhibits a GTPase activity, and a heterodimeric Gβγ, that is non-covalently bound to the Gα subunit. 20 mammalian Gα proteins are divided into four categories based on their sequence homology: Gαs, Gαi, Gαq and Gα12/13 [101].

The Gαs family is represented by adenylate cyclase stimulatory Gαs and the olfactory subunit Gαolf.

The Gαi family contains adenylate cyclase inhibitory Gαi1, Gαi2, Gαi3 and Gαio subunits. In addition, the Gαi family includes two retinal transducin Gα subunits (Gαt1, Gαt2), the taste Gα subunit gustducin (Gαgust) and Gαz. The Gαz subunit inhibits adenylate cyclase in pancreatic β-cells and has been reported to couple GPCRs to ion channels in neurons [102, 103].

The Gαq family, which activates phospholipase C, contains Gαq, Gα11, as well as Gα14 and Gα15.

The Gα12/13 family includes Gα12 and Gα13 subunits, responsible for Rho GTPase activation and regulation of cytoskeleton dynamics [104].

Modulating the activity of Gα subunits and/or G protein-coupled receptors can have beneficial applications in T2DM treatment. Elevated levels of cAMP in β-cells contribute to augmented insulin secretion and cell proliferation [105, 106]. Activation of Gαs subunits has similar effects on β-cell secretory functions and proliferation as inhibition of the Gαi action. Gαi inhibition by pertussin toxin (PTX) results in improved glucose stimulated insulin secretion and abolishes an inhibitory action of somatostatin on insulin release, both *in vivo* and in isolated rat islets [106]. Likewise, mice overexpressing the catalytic PTX subunit S1 in β-cells are resistant to high-fat diet induced hyperglycemia [107]. In addition, Gαz knockout mice have increased GSIS, β-cell mass and are protected from high-fat diet induced glucose intolerance [102]. The beneficial effects of the Gαz knockout are

linked to constitutively increased cAMP production in pancreatic islets. In addition, noradrenaline-induced inhibition of insulin exocytosis is abolished by a Gαi1/2 blocking protein in INS-1 β-cells [108]. Knockout of Gαio2 in mice has also been shown to enhance glucose stimulated insulin release and improve glucose tolerance [109]. However, mice with liver and adipose tissue deficiency of Gαi2 have decreased glucose tolerance, insulin sensitivity and IRS-1 phosphorylation in those organs, implying a positive effect of Gαi2 on the insulin action [110].

Incretins GLP-1 and GIP, acting through Gαs protein-coupled receptors, increase glucose-stimulated insulin secretion and β-cell mass [105, 111]. In addition, depletion of the GIP receptor in mice is associated with an impaired compensatory response to high fat diet, including inability to enhance β-cell proliferation and insulin secretion. Thus, GLP-1 and GIP analogues have been used to ameliorate T2DM symptoms, both in humans and animals [105, 112].

The Gαq family of G proteins also stimulates insulin secretion but in this case, adenylate cyclase is not involved. Gαq proteins activate phospholipase C, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Specific inactivation of Gαq and Gα11 in mouse pancreatic β-cells results in impaired glucose tolerance and insulin secretion [113]. Activation of cholecystokinin receptor B (CCKR), coupled to Gαq protein, by cholecystokinin and gastrin, has a stimulatory effect on basal and glucose-induced insulin secretion [114-116]. In addition, a combination therapy with epidermal growth factor (EGF) and gastrin increases β-cell mass and reverses hyperglycemia in diabetic NOD mice, an animal model of T1DM, whereas EGF and gastrin alone do not [117]. Similarly, a combination therapy of GLP-1 and gastrin, but not with GLP-1 and gastrin individually, restores normoglycaemia in NOD mice [118]. Interestingly, the GLP-1 receptor signals both through Gαs and Gαq proteins in hamster ovary cells [100].

In pancreatic β-cells, the Gα12/13 proteins, through Rho GTPase activation and reorganization of F-actin, may be involved in initiation of insulin exocytosis [119, 120].

There are 6 known human Gβ subunits (36 kDa) and 12 Gγ subunits (7-8 kDa) [121]. Gβγ dimers can stimulate adenylate cyclase isoforms 2, 4 and 7 while inhibiting adenylate cyclase isoforms 1, 5 and 6 [122]. In the context of β-cell physiology, Gβ5 knockout mice are characterized by decreased plasma glucose levels, impaired glucose clearance and increased plasma insulin [123]. The diversity of GPCR-mediated responses can be attributed to both receptor oligomerisation and a high number of possible G protein heterotrimer combinations.

1.5.3 Proteins modulating GPCR functions

Receptor activity modifying proteins (RAMPs) regulate trafficking and function of calcitonin receptor-like receptor (CRLR). The RAMP family consists of 5 members (RAMP1–RAMP5). Experiment in *Xenopus* and human cell cultures showed that when CRLR heterodimerizes with RAMP1 it can act as the calcitonin gene-related peptide 1 (CGRP1) receptor whereas when heterodimerizes with either RAMP2 or RAMP3 it can form adrenomedullin receptors AM1 and AM2 respectively [124]. In addition, CRLR membrane trafficking, ligand induced internalisation, recycling and desensitisation depend on forming complexes with RAMPs [124, 125]. CRLR forms stable heteromeric complexes with RAMPs, which are maintained throughout their life cycle [126]. Furthermore, RAMPs can interact with the calcitonin receptor (CTR), vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide receptor (VPAC1R), glucagon receptor, parathyroid hormone 1 and 2 receptors (PTH1R, PTH2R) and the calcium sensing receptor (CaSR) [127, 128].

GPCR localization and functions can also be modified by proteins containing post-synaptic density of 95 kDa (PSD95)-disc large-zona occludens (PDZ) domains. PDZ domains are responsible for protein-protein interactions by binding to specific PDZ recognition motifs located at the protein C-terminus [129]. GPCRs containing the PDZ-binding motif include serotonin receptors (5-HT2A, HTR2B, 5-HT2C), somatostatin receptor 2 (SSTR2) and parathyroid hormone 1 receptor (PTH1R). PTH1R can couple to either Gαs or Gαi proteins, depending on the interaction with

the PDZ domain-containing Na^+/H^+ exchanger regulatory factor 2 (NHERF2), an inhibitor of the renal NHE3 Na^+/H^+ exchanger. In the renal cells expressing NHERF2, PTH1R couples to the Gαi proteins predominantly, whereas in the cells lacking NHERF2, the receptor couples to the Gαs family proteins [130]. Moreover, the PSD95 protein, containing a PDZ domain, binds β1-adrenergic receptor (ADRB1) and suppresses receptor endocytosis while membrane-associated guanylate kinase inverted-2 (MAGI-2) protein upregulates ADRB1 internalization [131]. 5-HT2C serotonin receptor interaction with the multi-PDZ domain protein 1 (MUPP1) in the rat brain may be involved in GPCR signalosome assembling [132].

1.5.4 Signaling independent of G proteins

β2-adrenergic receptor (ADRB2) binding to the PDZ domain of NHERF1 prevents NHERF1 from inhibiting the NHE3 Na^+/H^+ exchanger activity in the kidney [133].

GPCRs can also activate non-G protein-dependent signaling through mobilization of β-arrestin [134]. After endocytosis, GPCRs can activate signaling pathways downstream of G proteins. The internalized GLP-1 receptor stimulates the activity of endosomal adenylate cyclase and increases glucose stimulated insulin secretion in rat pancreatic β-cells [135].

In addition, the β2-adrenergic receptor ligand ICI118551 functions both as an inverse agonist for Gαs-stimulated adenylyl cyclase and a Gαs-independent partial agonist for ERK1/2 kinase [136]. ERK1/2 activation by ICI118551 is inhibited in mouse embryonic fibroblasts lacking β-arrestin 1 and β-arrestin 2 proteins but can be restored by overexpression of β-arrestin 2, which suggests that β-arrestins mediate G protein-independent signaling. A similar mode of action was observed in the case of SR121463B, an arginine vasopressin receptor 2 (AVPR2) ligand [136]. β-arrestin-mediated activation of ERK1/2 involves formation of a multiprotein “signalosome”. Agonists of proteinase-activated receptor 2 (PAR2) promote formation of a protein complex consisting of internalized PAR2 receptor, β-arrestin

1, Raf-1 (a member of MAP kinase kinase kinase (MAP3K) family), and ERK1/2 [137].

1.6 WNT signalling

1.6.1 WNT proteins and their receptors

WNT1 proto-oncogene was first discovered in mouse mammary tumors [138]. Mammalian genomes contain 19 WNT genes, which encode proteins approximately 40 kDa in size. Even sponges and cnidarians, but not single cell organisms, harbor a few WNT genes, emphasizing the importance of WNT signaling in the evolution of multicellular species [139]. WNT proteins control tissue patterning, cell polarity, growth and proliferation [140, 141]. Moreover, WNT signalling plays a central role in development of skin, hair follicle, limbs, gastrointestinal tract, whiskers in mice as well as in intestinal stem cell maintenance [142].

WNT proteins exert their functions by activating heterodimeric receptor complexes, consisting of FZD (Frizzled) and single-pass transmembrane LRP5/6 subunits. 10 mammalian FZD receptors belong to the highly conserved Frizzled class of GPCRs [143]. WNTs interact both with the FZD and LRP5/6 subunits of the receptor complex. Furthermore, most of WNT proteins have the ability to engage multiple FZDs [144]. Likewise, 19 WNTs and 10 FZDs give 190 possible interactions, reflecting the complexity of WNT signaling.

1.6.2 Canonical WNT/β-catenin signaling

In the absence of WNT, the Axin-APC-GSK3 complex resides in the cytoplasm, where it phosphorylates β-catenin, resulting in subsequent β-catenin ubiquitination and degradation in the proteasome (Figure 1.5). GSK3 shows a constitutive phosphorylase activity. The adenomatous polyposis coli (APC) protein has the capacity to bind many β-catenin molecules, while Axin is responsible for binding the cytoplasmic domain of LRP5/6 [145].

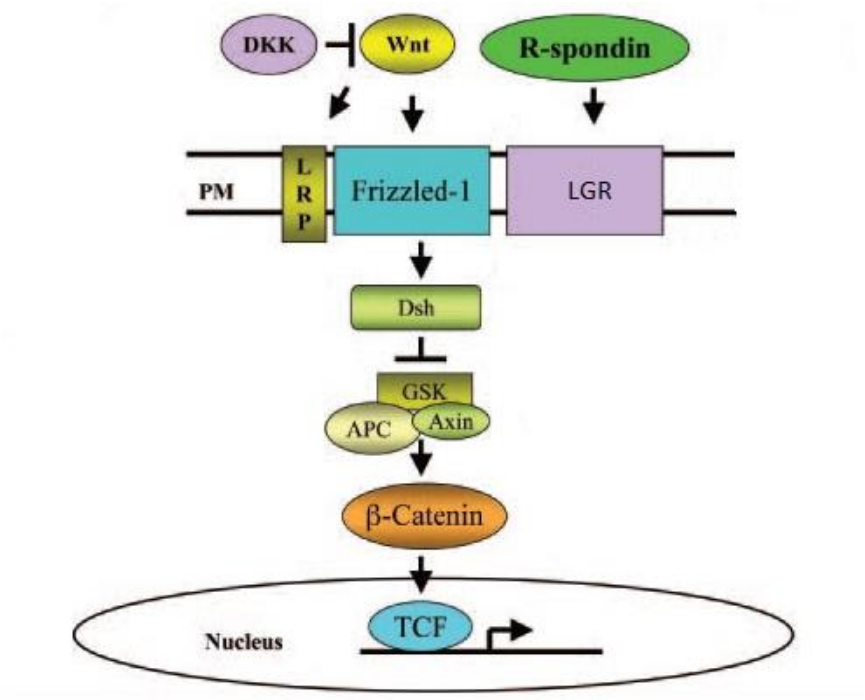

Figure 1.5. WNT/β-catenin signalling. The Axin-APC-GSK3 complex is responsible for the initiation of β-catenin degradation. WNT proteins activate the Frizzled-LRP receptor, which promotes deactivation of the Axin-APC-GSK3 complex by the cytosolic protein Dsh, leading to the β-catenin accumulation. R-spondins and their LGR receptors modulate the activity of the Frizzled-LRP complex. Dickkopf (DKK) proteins inhibit WNT binding to its receptor [146].

Binding of WNT to the FZD-LRP5/6 complex promotes association of Dishevelled (Dsh) protein with FZD and facilitates interaction between the cytoplasmic domain of LRP5/6 and Axin [147]. As a result, the Axin-APC-GSK3 complex falls apart, allowing β-catenin stabilisation and accumulation in the cytoplasm. Following translocation into the nucleus, β-catenin forms a complex with TCF transcription factor and activates target gene expression, including genes involved in the control of development and cell proliferation such as C-MYC oncogene, cyclin D1, and the metalloprotease matrilysin [148-151]. Dickkopf (DKK) proteins inhibit WNT signaling by binding LRP5/6 and disrupting FZD-LRP5/6 complex [152]. The role of R-spondins and their LGR receptors in the WNT signalling regulation is discussed in the next section.

Moreover, WNT/β-catenin signaling is implied in regulation of β-cell proliferation and insulin secretion in mice. The conditioned medium from L cells secreting

WNT3A increases proliferation of INS-1 cells by 2-fold, compared to the control proliferation level, in a β-catenin-dependent fashion [153]. Purified WNT3A protein also stimulates proliferation of both MIN6 and mouse islet cells [154]. In addition, activation of WNT/β-catenin pathway in isolated mouse islets results in augmentation of insulin secretion [153]. The WNT-mediated increase in β-cell proliferation is associated with upregulated expression of cyclins D1 and D2 in β-cells [153, 154]. The involvement of WNT signaling in the regulation of insulin secretion has also been confirmed by experiments on LRP5 knockout mice, which have impaired glucose-stimulated insulin secretion [155].

1.6.3 Regulation of the WNT pathway by R-spondins

R-spondin 1 (Roof plate specific-spondin 1) was first discovered in the roof plate of developing mouse embryos [156]. There are four members of the R-spondin protein family, RSPO1-4. R-spondins are approximately 30 kDa in size and have been shown to be secreted proteins (Figure 1.6) [146].

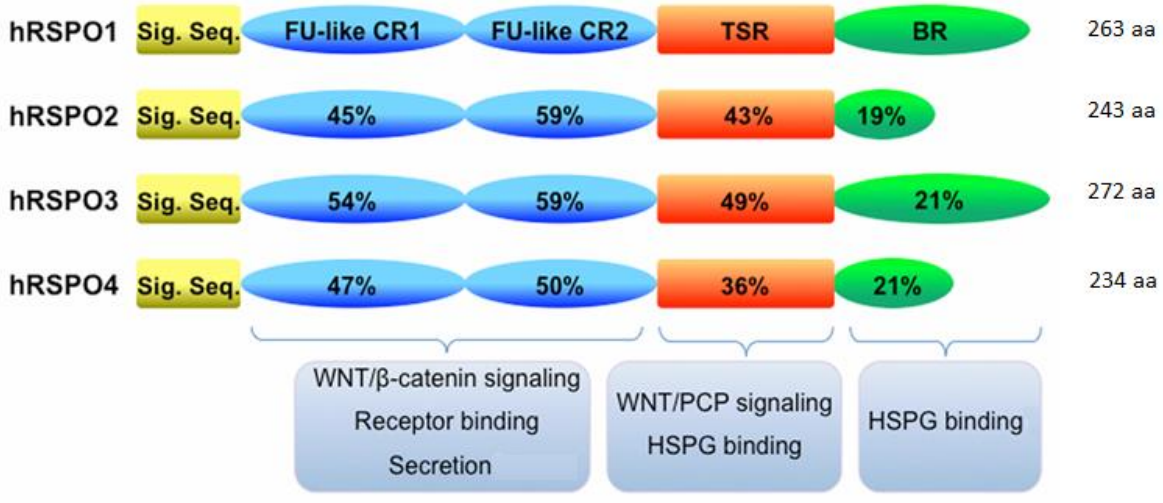

Figure 1.6. Human R-spondin protein family. Sig. Seq. – signalling sequence, FU-like CR – furine-like repeats, TSR – thrombospondin repeat, BR – basic amino acid-rich domain, HSPG – heparan sulphate proteoglycan. Sequence homology within the protein domains is expressed as the percentage of identical amino acids [157].

However, R-spondins have the capacity to bind heparan sulphate proteoglycans (HSPG) of the cell surface and extracellular matrix. Consequently, this feature may hinder *Xenopus* RSPO2 and mouse RSPO1 detectability when they are secreted upon overexpression in human T293 cells into culture medium [156, 158]. On the contrary, overexpressed human RSPO1 is efficiently secreted from T293 cells [159].

RSPO1 regulates gonadal sex determination and skin differentiation. RSPO1 gene mutations in humans lead to XX male sex reversal [160]. R-spondin 1 protein has a mitogenic effect on intestinal crypt epithelial cells in mice [161]. In addition, the RSPO1 protein stimulates proliferation and inhibits apoptosis of MIN6 cells, as well as enhances glucose-independent insulin secretion [162]. Nonetheless, experiments on RSPO1 knockout mice revealed that RSPO1 has a negative effect on β-cell neogenesis from pancreatic ductal cells [163]. RSPO2 plays a key role in development of limbs, lungs and hair follicle in a WNT/β-catenin pathway-dependent manner [164-166]. RSPO3 is involved in vascularization of the developing placenta [167]. Mutations in the RSPO4 gene cause anonychia, a mild disorder characterized by the absence of fingernails and toenails [168, 169]. R-spondins 1-4 are expressed in mouse and human islets [162, 170]. However, only expression of RSPO1, RSPO3 and RSPO4 have been detected in MIN6 cells [162]. Furthermore, RSPO1, RSPO3 and their receptor LGR4 are expressed in the hypothalamus [171]. RSPO1 and RSPO3 injection in to the mouse third brain ventricle, bordering the hypothalamus, results in inhibition of food intake [171]. In the hypothalamus, LGR4 colocalises with neuropeptide Y, which stimulates food intake [172]. These observations, and the fact that insulin upregulates RSPO1 and RSPO3 gene expression in the hypothalamus, suggest a role for R-spondins in the regulation of energy homeostasis [171].

R-spondins associate with LGR (leucine rich repeat containing G protein coupled) 4-6 receptors, mediating the enhancement of canonical WNT and WNT/PCP pathways signalling [173, 174]. The pro-proliferative action of RSPO1 is blocked in isolated mouse intestinal crypt epithelial cells of LGR4/LGR5 knockout mice, but

can be rescued by WNT3 overexpression [174]. LGR4-6 are approximately 100 kDa in size, share 50% amino acid sequence homology and belong to the GPCR class A (rhodopsin-like) [175]. Moreover, LGR4 and LGR5 are expressed in human islets [170]. Although mouse LGR4 has not been directly demonstrated to associate with Gαs proteins its overexpression in osteoblasts results in an increase in intracellular cAMP levels and upregulation of cAMP-dependent PKA [176]. Notably, stimulation of mouse LGR4 and LGR5, stably expressed in HEK293 cells, by mouse RSPO1 and RSPO2 proteins at various concentrations does not lead to activation of Gαs, Gαq and Gαi protein signaling pathways [173].

R-spondins interact with extracellular domains of LRP5/6 and FZD and modulate positively WNT/β-catenin signaling [164, 177] (Figure 1.7). RSPO1, RSPO2 and RSPO3 are more potent at upregulating WNT pathway than RSPO4 [178].

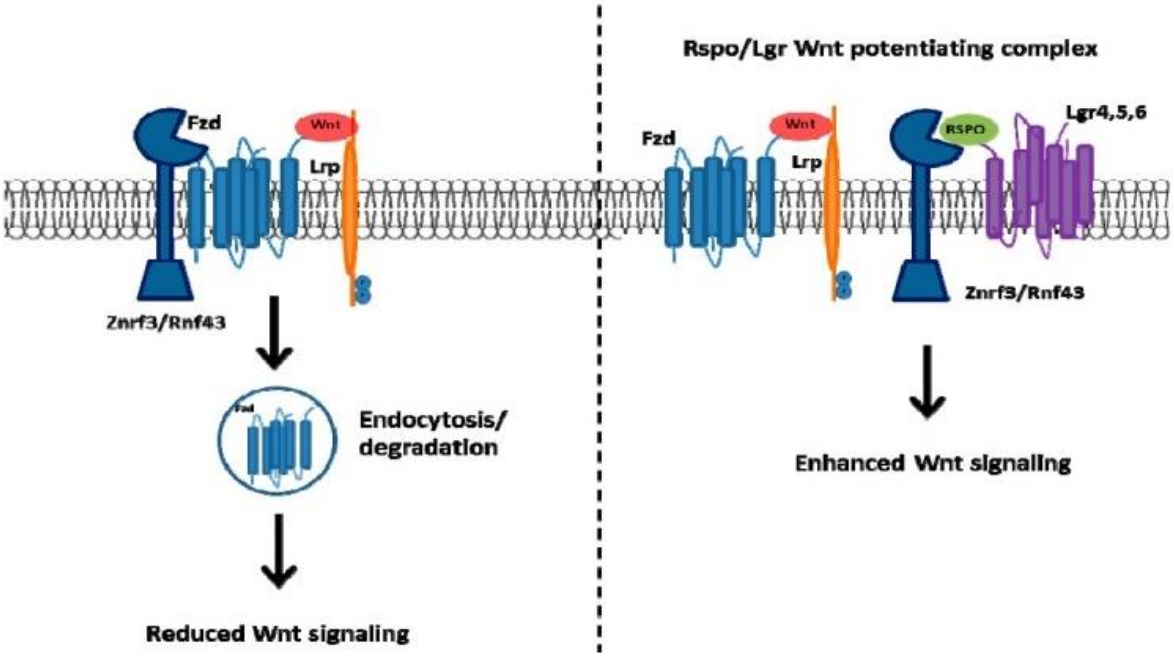

Figure 1.7. Regulation of WNT signalling by RSPO proteins. R-spondins stabilise the FZD-LRP complex and enhance WNT signalling. R-spondins bind LGR and interact with the FZD-LRP complex, which prevents Znrf3/Rnf43 ubiquitin ligases from the initiation of FZD-LRP degradation [179].

Znrf3/Rnf43 ubiquitin ligases bind the FZD-LRP receptor complex, causing polyubiquitination of lysines in the cytoplasmic domains of FZD, followed by endocytosis and receptor degradation (Figure 1.7). RSPO1 has been shown to interact with the extracellular domain of Znrf3 and to mediate association of Znrf3 with LGR4, leading to membrane clearance of Znrf3 and enhancement of WNT signalling in human HEK293 cells [180]. R-spondin proteins increase endogenous levels of FZD proteins in HEK293 cells [180]. Moreover, human RSPO1 protein potentiates canonical WNT signalling by inhibiting internalization of LRP6 in HEK293 cells and stimulation of endogenous LRP6 phosphorylation in mouse embryonic fibroblasts (MEFs) [159, 181].

In addition, R-spondins bind syndecans, proteoglycans controlling adhesion, differentiation and proliferation [182]. Syndecans are single trans-membrane domain proteins, carrying three to five heparan sulphate or chondroitin molecules, which confer the ability to associate with various growth factors and chemokines [183, 184]. An interaction of RSPO3 with LGR4, LGR5 and syndecan 4 activates the non-canonical WNT/planar cell polarity (PCP) pathway, involved in gastrulation and embryogenesis [185, 186]. RSPO3 binding to syndecan 4 and LGR4/5 promotes endocytosis of the FZD receptor complex and signaling initiation, but does not involve LRP5/6 [185]. Syndecan 4 directly interacts with FZD and Dsh and mediates translocation of Dsh to the plasma membrane [187]. Subsequently, a complex of Dsh, Rho and Daam1 is formed in the cytoplasm. Daam1 mediates the Dsh and Rho interaction and Rho activation by guanine nucleotide exchange factors (GEFs), which catalyse an exchanged of Rho-bound GDP to GTP [188]. Activated Rho initiates the activity of Drosophila Rho-associated kinase (Drok), resulting in myosin phosphorylation and rearrangement of the cytoskeleton [189]. The Rho activity can be switched off by GTPase-activating proteins (GAPs) [190].

There are many signalling pathways involved in the regulation of β-cell functions during pregnancy. Several of those pathways are discussed in this chapter but our knowledge of the β-cell adaptive responses is still limited. In the next part of this thesis I will be looking at some of these mechanisms in more detail.

1.7 Aims and objectives

The overall aim of this thesis was to study mechanisms regulating β-cell mass expansion, using pregnancy in mice as an experimental model in which the β-cell mass increases during gestation and returns to normal levels post-partum. The mechanisms underlying this adaptation are not well understood, although placental signals are thought to be involved. Reduction of β-cell mass or failure to expand β-cell mass during pregnancy are hallmarks of T2DM and GDM respectively. Therefore, targeting mechanisms regulating β-cell mass expansion may help to develop new therapies for T2DM.

The hypothesis tested in this thesis will be:

1. Pregnancy and lactation stimulate β-cell proliferation, with selective loss of new β-cells post-partum.
2. Novel proteins secreted by the placenta can enhance β-cell function during pregnancy.

The first objective of the project was to analyse changes in the β-cell mass during pregnancy, and post-partum. The second objective was to quantify the expression of islet β-cell GPCRs and their placental ligands to identify novel placental signals potentially involved in β-cell adaptation to pregnancy. The third objective was to examine the effects of one of the novel placental signals, RSPO4, on β-cell function.

Chapter 2

Materials and methods

Chapter 2

2.1 Animals

2.1.1 Mice for pancreatic islet BrdU staining

Female CBA/Ca mice (MRC, UK) at 8 weeks of age were used. Mice were kept under a light–dark cycle of 12 h and were fed ad libitum with a normal diet. For pregnant mice, females were housed with a male and checked daily for the presence of a vaginal plug. The day a vaginal plug was observed was designated day 0 of pregnancy. Mouse gestational time is 19-21 days. Mice were given 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, which was incorporated into the DNA of proliferating cells, in their drinking water as a 1 mg/ml solution. Pregnant mice were given BrdU solution during gestational days 8-17 (Figure 2.1) and terminated on day 18. Another group of pregnant mice were given BrdU solution during gestational days 8-17 followed by water during days 1-10 post-partum (maintained with pups).

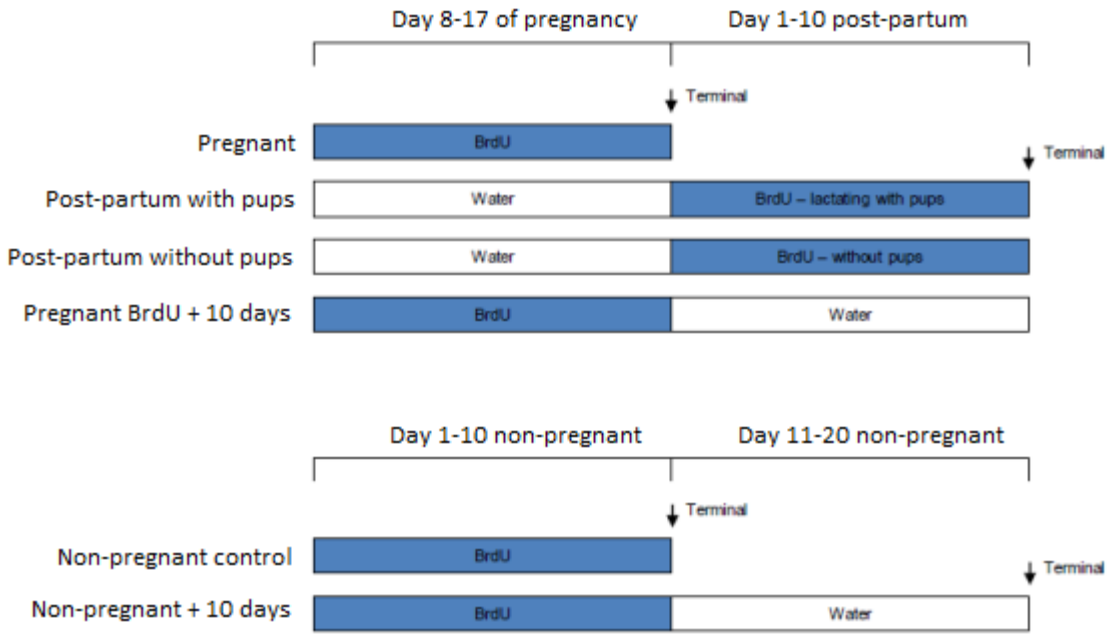

Figure 2.1. Summary of BrdU administration regimes in different experimental groups.

Post-partum mice were given BrdU solution for days 1-10 post-partum and were maintained with or without pups during that time. Non-pregnant control mice were given BrdU solution for 10 days or BrdU solution for 10 days, followed by water for a further 10 days (Figure 2.1). Mice were culled one day after finishing the treatment.

2.1.2 Mice for placental GPCR ligand secretome and GPCRome analysis

Female CD1 mice (Charles River, UK) at 8 weeks of age were used. Mice were kept under a light–dark cycle of 12 h and were fed ad libitum with a normal diet. Females were housed with a male and checked daily for the presence of a vaginal plug. The day a vaginal plug was observed was designated day 0 of pregnancy. On gestational day 12 or 18 mice were culled, followed by isolation of placentas and pancreatic islets.

2.2 Immunofluorescence staining

Mouse β-cell proliferation was measured using immunohistochemical (IHC) staining of pancreatic sections labelled with BrdU. Mouse pancreas samples were fixed with 4% paraformaldehyde/PBS solution overnight, embedded in paraffin wax and sectioned in 5 μm longitudinal sections of paraffin blocks. Details of immunostaining buffers are shown in Table 2.1. Pancreatic sections were rehydrated by washing with xylene (VWR) and decreasing concentration of ethanol (100%, 95% and 70% ethanol) (VWR), then washed with water and TBS, followed by permeabilization with 2M hydrochloric acid (VWR) for 20 min at 37°C. After washing with water and TBS, the sections were permeabilized with 0.05% trypsin solution (Sigma) for 15 min at 37°C and then washed with TBS. The sections were blocked with the blocking buffer for 15 min at room temperature and incubated with mouse anti-BrdU (1:100 dilution, Sigma) and guinea pig anti-insulin (1:200 dilution, Dako) primary antibodies for 2 h at 37°C. After incubation with the primary antibodies, the sections were washed with TBS and stained with FITC-labelled anti-mouse (1:100 dilution, Jackson ImmunoResearch Laboratories) and

Alexa Fluo 594-labelled anti-guinea pig (1:100 dilution, Jackson ImmunoResearch Laboratories) antibodies for 1 h at room temperature. Sections were washed with TBS, mounted and visualized using a fluorescent microscope (Nikon Eclipse TE2000-U). Fluorescent images of islets were acquired in x 200 magnification, using NIS Elements BR20 software (Nikon).

Table 2.1. Composition of buffers for IHC.

Buffer	Composition
TBS (Sigma)	50 mM Tris, 150 mM NaCl, pH 7.6
PBS (Sigma)	137 mM NaCL, 2.7 mM KCL, 10 mM $Na_2HPO_4 * 2H_2O$, 2 mM KH_2PO_4, pH 7.4
Blocking buffer (Sigma)	0.25% BSA, 0.25% Triton X100 in PBS

2.2.1 Analysis of pancreatic β-cell proliferation

For each islet image the number of BrdU labelled β-cells and the total number of β-cells were counted using ImageJ program. A minimum of 3 sections were analysed per animal and a minimum of 10 islets were counted per section. The results were expressed as the percentage of total β-cells that were positively stained with BrdU for each animal.

2.2.2 Analysis of pancreatic β-cell area

The cross-sectional area of individual β-cells was analysed on the same sections as those used for the proliferation analysis. The total β-cell area was calculated by tracing around the perimeter of the islet tissue and measuring the area by the ImageJ software (MicroSmarts LLC). A total number of β-cells was counted and the β-cell area divided by the number of β-cells to give the mean cross-sectional area of the individual β-cell. This measurement is an estimate of individual β-cell size as it does not take into account the fact that not all of the nuclei of β-cells in the section would be present. Therefore, this measurement is likely to overestimate the actual β-cell cross-sectional area. Nonetheless, it is a useful

method of comparing β-cell size between groups of animals analysed in the same way.

2.3 Isolation of mouse pancreatic islets

Female CD1 mice (Charles River) were culled by cervical dislocation followed by surgical opening of the abdominal cavity. The common bile duct was clamped at the ampulla of Vater and cannulated with a 27 gauge needle [191]. 3 ml of ice cold collagenase solution (3 mg/ml, Sigma) was injected to inflate the pancreas, which was then extracted, placed into a 50 ml centrifuge tube and kept on ice. The isolated pancreas was incubated for 15 min in a water bath at 37°C to digest the tissue. The digestion was terminated by re-suspending the digest with Minimum Eagles Medium (MEM) (Sigma), containing 10% Newborn Calf Serum (NCS) (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma). The tubes containing the pancreatic isolate were shaken by hand and centrifuged at 340 g, 10°C, for 15 min. The suspension was then filtered through a collector tissue sieve and spun in a centrifuge tube at 340 g, 10°C, for 1 min. The tissue pellet was then resuspended in 25 ml of Histopaque-1077 (Sigma). 10 ml of MEM was pipetted on the top of the Histopaque-1077 layer followed by a gradient centrifugation of at 700 g, 10°C, for 24 min. Isolated islets were recovered from the interface between the Histopaque-1077 and MEM, washed three times with MEM, placed on a plastic petri dish before being hand-picked under a dissecting microscope and washed with Roswell Park Memorial Institute (RPMI) medium (Sigma), supplemented with 10% NCS, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The islets were incubated overnight at 37°C (95% air/ 5% CO_2) before being used for total RNA isolation.

2.4 RNA isolation

The quality of isolated RNA is crucial for downstream applications, including complementary DNA (cDNA) synthesis and cDNA amplification using polymerase chain reaction (PCR). Mouse pancreatic islet, placenta and pancreatic MIN6 β-cell

total RNA was isolated using Qiagen RNeasy Mini kit, according to the manufacturer's instructions. RLT lysis buffer, RNase-free water, wash buffers RW1 and RPE were included in the kit package.

2.4.1 Total RNA isolation from mouse pancreatic islets and pancreatic MIN6 β-cells

Isolated mouse pancreatic islets (150-250 islets/animal/isolation) or a maximum of 10^7 pancreatic MIN6 cells were resuspended in 600 µl of RLT lysis buffer. Subsequently, the lysate was transferred into a homogenization Qiagen QIAshredder spin column and centrifuged at room temperature for at 7000 g for 3 min. 600 µl of 70% ethanol was added to the supernatant and mixed by pipetting. 700 µl of the homogenate was then loaded into a spin mini column and centrifuged at 7000 g for 15 s. After discarding flow through, 350 µl of RW1 wash buffer was pipetted into the column, followed by centrifugation at 7000 g for 15 s. Flow through was discarded and DNase solution (10 µl DNase in 70 µl of RDD buffer, Qiagen) was then applied into column and incubated at room temperature for 15 min. The incubation was stopped by adding 350 µl of RW1 buffer into the column, which was followed by centrifugation (7000 g for 15 s) and removal of flow through. The column was washed with 500 µl of RPE buffer at 7000 g for 15 s followed by adding 500 µl of RPE buffer into the column and centrifugation at 7000 g for 2 min. The column was then transferred into a sterile 1.5 ml tube and RNA was eluted by adding 30 µl of RNase free water before centrifugation at 7000 g for 1 min. RNA samples were quantified and immediately converted into cDNAs.

2.4.2 Determination of RNA concentration

Total RNA concentration was determined using the ND1000 spectrophotometer (Nanodrop). The device measured 260 and 280 nm absorbance (A_{260} and A_{280}) in 1.5 µl of the sample RNA solution. The A_{260} value was automatically converted into RNA concentration expressed as µg/µl. The A_{260}/A_{280} ratio was used to evaluate purity of isolated RNA. RNA samples with A_{260}/A_{280} ratios between 1.8

and 2.2 were considered sufficiently pure and were used for further experiments. Sample RNAs were converted immediately into cDNA.

2.5 cDNA synthesis

mRNAs were converted into cDNA by the process of reverse transcription, using a High Capacity Reverse Transcription Kit (Applied Biosystems). All of the reagents used were provided with the kit. A reaction mixture was prepared as described in Table 2.2.

Table 2.2. Reverse transcription reaction mixture.

Component	Volume [µl]
10x Reverse Transcription (RT) buffer	2.0
20x Deoxynucleotide (dNTP) mix	0.8
10x Random Primers	2.0
MultiScribe Reverse Transcriptase	1.0
Nuclease-free water	4.2
Total per reaction	10

10 µl of the sample RNA solution (0.5-1 µg of RNA) was mixed with 10 µl of the reaction mixture in a 0.2 µl PCR tube. The tube was spun briefly and placed in a thermocycler (Bio-Rad T100). Reverse transcription was conducted using thermal cycler program conditions summarized in Table 2.3. cDNA samples were stored at -80°C.

Table 2.3. Reverse transcription thermal cycler program conditions.

	Step 1	Step 2	Step 3	Step 4
Temperature [°C]	25	37	85	4
Time	10 min	120 min	5 min	∞

2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

Real-time quantitative reverse transcription PCR is an efficient method of accurate gene expression quantification [192]. The PCR technique allows DNA sequence amplification *in vitro* [193]. In this technique, DNA polymerase synthesises complementary strands of the DNA template by extending sequence-specific oligonucleotide primers. DNA amplification is achieved in successive cycles of denaturation of double stranded DNA, annealing of primers to single stranded DNA and extension of newly synthesised DNA strands. Real-time PCR utilizes fluorescent SYBR Green hybridization probe, which emits signal only when bound with double stranded DNA, to monitor amplification in real time (Figure 2.2).

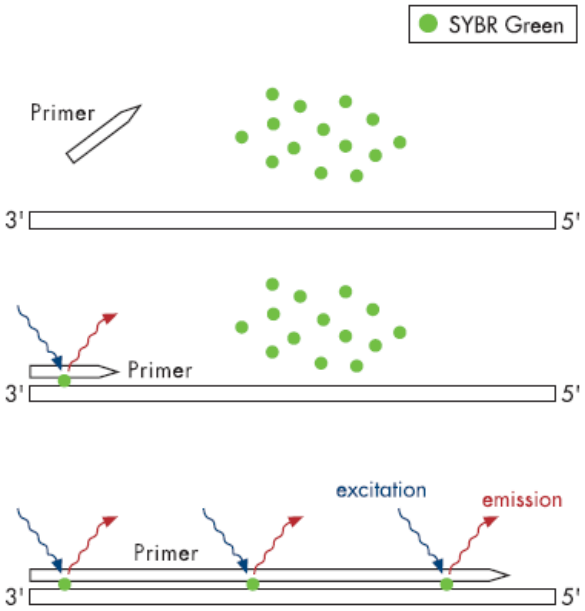

Figure 2.2. SYBR Green-based product detection in real-time PCR. The SYBR Green probe hybridizes with double stranded DNA and emits green light when excited with blue light during the DNA amplification process, enabling monitoring of amplification [194].

Fluorescent signal intensity at the beginning of the exponential phase of cDNA amplification is proportional to the amount of target cDNA and thus the target gene expression level in the sample.

2.6.1 Normalization against mouse housekeeping gene

Normalization against the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference housekeeping gene is necessary to determine the optimal cDNA concentration used for gene screening. A cDNA concentration, which produced the cycle threshold (Ct) value of 20, was used for gene expression screening and quantification, allowing efficient analysis of a few hundred genes in a single cDNA sample. The Ct value is defined as the number of cycles required for reaction fluorescence to exceed the background threshold level. The lower the Ct, the higher the amount of target cDNA in the sample. The stock cDNA solution (in triplicates) was diluted 1 in 10 and mixed in a 96-well plate with the QuantiFast SYBR Green Master Mix (Qiagen), water and the 5x GAPDH QuantiTect primer assay (Qiagen) (Table 2.4).

Table 2.4. Reaction volumes for housekeeping gene expression normalization.

Reagent	Volume / well [µl]
SYBR Green master mix	5
cDNA (diluted 1 in 10)	1
water	2
5x GAPDH QuantiTect primer assay	2
Total volume	10

The plate was centrifuged for 1 min at 500 g, placed in a Roche LC480 light cycler and run using real-time quantitative PCR program conditions shown in Table 2.5. Results were analysed using Roche LC480 light cycler software to determine the dilution factor required to generate a GAPDH gene Ct value of 20 for each cDNA sample. Figure 2.3 presents an example of GAPDH normalization results in pancreatic islet cDNA samples of non-pregnant mice. Individual melting curves, with the same single temperature peaks, correspond to the same DNA sequence amplified in the reaction.

Table 2.5. Real-time quantitative PCR program conditions.

Step	Target Temp [°C]	Acquisition mode	Time	Ramp rate [°C/s]	Acquisitions per °C	Cycles	Analysis Mode
Pre-incubation	95	None	5 min	4.4	-	1	None
Amplification	95	None	10 s	4.4	-	40	Quantification
	60	Single	30 s	2.2	-		
Melting curve	95	None	5 s	4.4	-	1	Melting Curves
	65	None	1 min	2.2	-		
	97	Continuous	-	0.11	5		
Cooling	40	None	30 s	2.2	-	1	None

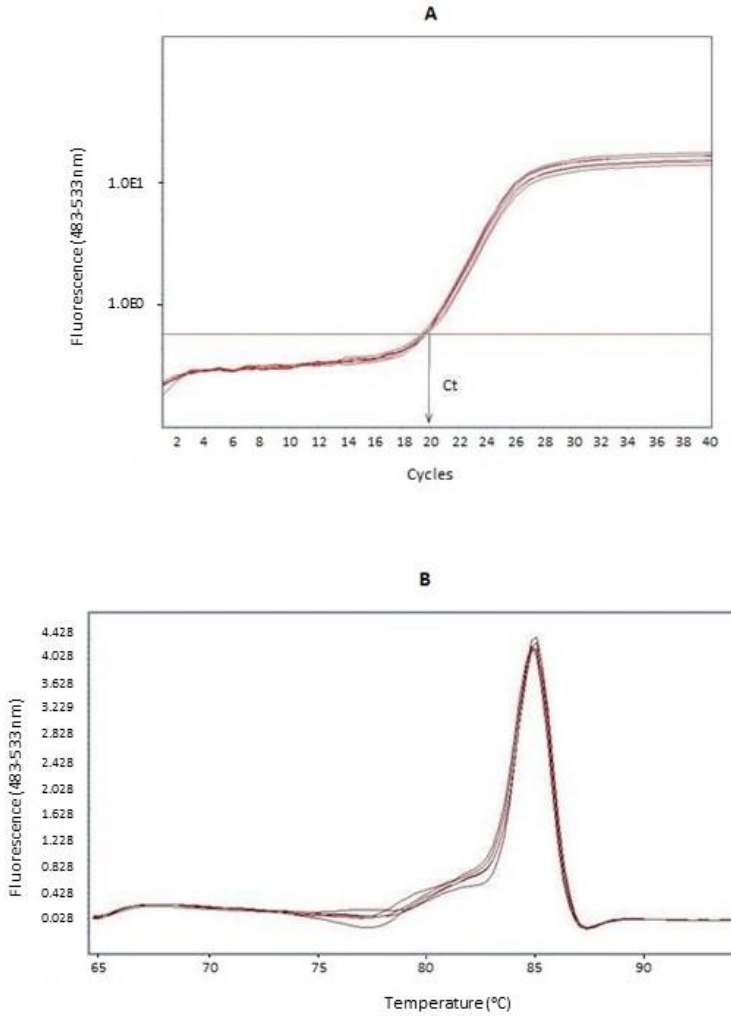

Figure 2.3. GAPDH normalization in mouse islet cDNA samples of 8 non-pregnant mice. Sample cDNA was diluted to produce an amplification reaction Ct value of 20. A – amplification curves, B – melting curves, Ct – cycle threshold.

2.6.2 Screening for mRNA expression

Quantitative RT-PCR was used to screen placental extracts for the expression of mRNAs encoding 126 potential islet GPCR ligands “secretome” on gestational day 12 and 18 as well as 342 GPCR mRNAs in pancreatic islets from non-pregnant mice, and from islets isolated from gestational day 12 and day 18 animals (8 animals in each group). In addition, expression of selected genes was analysed in mouse pancreatic MIN6 β-cells to confirm β-cell specific expression. cDNAs from 8 mice in each group of animals was pooled and diluted to produce a GAPDH gene Ct value of 20. A single pooled cDNA replicate for each screened primer pair (QuantiTect primer assay, Qiagen) was analysed. 94 gene QuantiTect primer assays and a duplicate of GAPDH gene QuantiTec primer assay were analysed in a 96-well plate. A reaction mixture was prepared according to Table 2.6. Details of primers used for the GPCR ligand and GPCR gene expression analysis are presented in Appendix II. Screening qPCR reaction products were analysed by agarose gel electrophoresis to confirm amplicon size (section 2.6.3).

Table 2.6. Reaction volumes for screening of mouse placental GPCR ligand genes and pancreatic islet GPCR genes.

Reagent	Vol reagent / well [µl]	Vol reagent / plate [µl]
SYBR master mix	5	528
cDNA	1	105.6
Water	2	211.2
5x QuantiTect primer assay	2	-
Totals	10	844.8

Only genes producing a specific product in the screening reaction, characterised by a single product melt curve peak and an agarose gel electrophoresis product band (section 2.6.3), corresponding to the size given in the QuantiTect primer assay specification, were quantified (section 2.6.4).

2.6.3 DNA agarose gel electrophoresis

The molecular size of screening reaction products was analysed by agarose gel electrophoresis. After the gene expression screening, 10 µl of each product, mixed with 2 µl of the 6x loading buffer (Promega), was loaded onto a 2% agarose gel, containing 5 µg/ml ethidium bromide (Sigma). Electrophoresis was conducted in Tris/borate/EDTA (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.6) buffer (TBE) (Sigma) using a 100 bp DNA ladder (Promega) as a DNA size marker (50-100 V, 1-2 h). DNA was visualized using a UV transilluminator (Syngene). Genes generating a single product band of the correct molecular size, in accordance with the QuantiTect primer assay specification, were quantified.

2.6.4 Gene expression quantification

Expression of genes, which produced positive results in the expression screening analysis, was quantified. Relative expression of target genes was determined by the $2^{-\Delta\Delta Ct}$ method, using mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a reference housekeeping gene [195]. Each of the gene primer assays was analysed in one replicate for each of the 8 mice per group. Reaction mixtures and the light cycler programme were used as described in Tables 2.4 and 2.5 of the house-keeping gene normalization chapter (section 2.6.1). Expression of target genes relative to the GAPDH gene, quantified in duplicate, was calculated using the formula shown in Figure 2.4.

$$\text{Relative expression} = \frac{2^{-\text{Ct gene of interest}}}{2^{-\text{Ct GAPDH}}}$$

Figure 2.4. Calculation of relative gene expression. Ct – threshold cycle.

2.7 Analysis of RSPO4 protein concentration in mouse plasma during pregnancy

RSPO4 protein levels were determined in plasma samples of non-pregnant, gestational day 12 and gestational day 18 CD1 mice, using a colorimetric mouse R-spondin 4 (RSPO4) ELISA kit (Cusabio), according to the manufacturer's instructions. Standard, standard diluent, biotin-antibody, HRP-avidin, washing buffer, substrate and stop solutions were provided with the kit. Mice were culled by cervical dislocation followed by cardiac puncture blood retrieval. Heparin was added as an anticoagulant into each sample, which was then centrifuged at 1000 g, 4°C, for 15 min. Plasma supernatants were collected and stored at -80°C for further analysis. After thawing, plasma samples were centrifuged (1000 g, 4°C, 15 min), 100 ul of each sample and standard were added per well and incubated at 37°C for 2 h. Standards were prepared by a serial dilution of the concentrated standard stock solution in the standard diluent (2200-34.4 pg/ml) Subsequently, 100 ul of the biotin-antibody were added to each well and incubated at 37°C for 1 h. Wells were then washed 3 times with the washing buffer. 100 ul of horseradish peroxidase (HRP)-avidin were added to each well and incubated for 1 h at 37°C. Wells were then washed 5 times with the washing buffer and 90 ul of the substrate were added to each well. After 15 min of incubation at 37°C, 50 ul of the stop solution were added to each well. The optical density of the reaction product solution in each well was determined by a micro plate reader (Turner Biosystems) at 450 nm. A standard curve was prepared and sample RSPO4 concentrations were calculated from the curve.

2.8 Culturing MIN6 β-cells

The insulinoma MIN6 cell line was derived from a transgenic mouse expressing the simian virus 40 T antigen in pancreatic β-cells [196]. MIN6 cells express insulin and T antigen and exhibit morphological characteristics of pancreatic β-cells. Moreover, those cells retain physiological features of normal mouse islet β-cells including glucose-inducible insulin secretion, glucokinase activity as well glucose

transport and utilization [197]. High passage MIN6 cells (60-70 passages) have impaired insulin secretion as well as reduced glucose and lipid oxidation [198]. Therefore, MIN6 cells of up to 40 passages should be used for β-cell research as those cells retain normal β-cell functions [198]. In this work, MIN6 cells of 30-35 passages were used for all the experiments.

MIN6 β-cells were cultured in growth medium, Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose, at 37°C (5% CO_2, 95% air) in a tissue culture incubator. Details of culture media used for MIN6 β-cell culturing are presented in Table 2.7. Growth medium was changed every 2-3 days. MIN6 cells were maintained as monolayers attached to the bottoms of 75 cm^3 tissue culture flasks (ThermoScientific). The cells were passaged after reaching 70-80% of confluence. To detach cells from the flask bottom surface, growth medium was removed and cells were washed with PBS which was followed by addition of 0.05% Trypsin/0.02% EDTA (Sigma) solution (3 ml). The flask was then incubated for 3 min at 37°C in a tissue culture incubator. The cells were resuspended in 7 ml of growth medium and centrifuged at 500 g for 3 min. The supernatant was then discarded and the cell pellet was resuspended in DMEM growth medium. The cells were either used in experiments or to inoculate a fresh tissue culture flask. MIN6 cells of 30-35 passages were used for experiments.

2.9 MIN6 β-cell proliferation assay

The effect of mouse R-spondin 4 (RSPO4) protein (Cusabio) on β-cell proliferation was evaluated using a colorimetric ELISA assay (Roche) based on BrdU incorporation into DNA. BrdU was incorporated into the DNA of proliferating cells. The method uses an anti-BrdU antibody conjugated with peroxidase (anti-BrdU-POD) which binds to the incorporated BrdU. Peroxidase catalyzes oxidation of a substrate, in the presence of hydrogen peroxide, into a detectable coloured product. FixDenat, BrdU, anti-BrdU-POD, wash and substrate solutions were included in the assay kit.

MIN6 cells were detached from the culture flask by incubation with 0.05% Trypsin/0.02% EDTA solution (Sigma) at 37°C for 3 min in a tissue culture incubator. The cell number and viability were measured in the cell suspension using Celltess™ automatic cell counter (Invitrogen). 20000 cells were plated per one well of a 96-well cell culture plate and cultured in 200 µl of the DMEM growth medium overnight (18 h) at 37°C (5% CO_2, 95% air) in a tissue culture incubator. Details of culture media used for the MIN6 cell proliferation assay are presented in Table 2.7. Following the overnight incubation, growth medium was replaced with starving DMEM medium and cultured at 37°C for 48 h. After 48 h medium was replaced in each well with 200 µl of different media, depending on the treatment group: serum-free treatment DMEM medium containing RSPO4 protein, serum-containing DMEM medium containing RSPO4 protein, serum-free DMEM medium without RSPO4 protein or serum-containing DMEM medium without RSPO4 protein (6 wells per treatment) (Table 2.8). Four RSPO4 protein concentrations were tested: 35 pM, 350 pM, 3.5 nM and 35 nM (Table 2.8). Cells were cultured at 37°C for 48 h. After 48 h, 10 µl of BrdU stock solution (10 µM) was added to each well and incubated at 37°C for 3 h in a cell culture incubator. Following BrdU labelling incubation, medium was removed from the plate, 200 µl of the FixDenat solution was added to each well and incubated for 30 min at room temperature. The FixDenat solution was then removed from the plate and 100 µl of the anti-BrdU-POD solution was added to each well. After 90 min of incubation at room temperature, the anti-BrdU-POD solution was removed and the wells were washed three times with 200 µl of the washing solution. 100 µl of the substrate solution was added to each well followed by 5 min incubation of the plate on a plate shaker at room temperature. Addition of 25 µl of 1N H_2SO_4 solution (VWR) into each well stopped substrate conversion into a detectable coloured reaction product. Product absorbance was read at 450 nm using Chameleon^{TM}V (Hidex) plate reader.

Table 2.7. Culture media used for the MIN6 cell proliferation assay.

Medium	Composition
DMEM growth medium (Sigma)	25 mM Glucose, 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol
DMEM starving medium (Sigma)	2 mM Glucose, 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol
Serum-free DMEM, for RSPO4 protein treatment (Sigma)	20 mM Glucose, 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol
Serum-containing DMEM, for RSPO4 protein treatment (Sigma)	20 mM Glucose, 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol

Table 2.8. Treatment map of the MIN6 cell proliferation assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		No RSPO4	35 pM RSPO4	350 pM RSPO4	3.5 nM RSPO4	35 nM RSPO4	No RSPO4	35 pM RSPO4	350 pM RSPO4	3.5 nM RSPO4	35 nM RSPO4	
C												
D												
E												
F												
G												
H												
		Serum-free DMEM					Serum-containing DMEM					

2.10 MIN6 β-cell apoptosis assay

The effect of mouse RSPO4 protein on β-cell apoptosis was analysed using Caspase Glo® 3/7 assay kit (Promega), based on luminescence. The kit assesses apoptosis by measuring the activity of caspase 3 and caspase 7, which are activated in the early stages of apoptosis. Caspase 3/7 activate a luminogenic substrate, generating amino-luciferin, which is then oxidised by luciferase to generate a luminescent signal. The luminescent signal intensity is proportional to the caspase 3/7 activity. Caspase Glo buffer and Caspase Glo substrate were provided with the kit.

MIN6 cells were detached from the culture flask by incubation with 0.05% Trypsin/0.02% EDTA solution (Sigma) at 37°C for 3 min. The cell number and viability were measured in the cell suspension using Celltess™ automatic cell counter (Invitrogen). 20000 cells were plated per one well of a 96-well cell culture plate and cultured in 200 µl of the DMEM growth medium overnight (18 h) at 37°C (5% CO_2, 95% air) in a tissue culture incubator. Table 2.9 shows details of culture media used for the MIN6 cell apoptosis assay. Following the overnight incubation, growth medium was removed from the plate and the plate wells were washed with 200 µl of PBS (Sigma). PBS was replaced with 100 µl of a treatment solution in each well (2% serum DMEM with or without RSPO4 protein and cytokines) as presented on a plate treatment map in Table 10. Four RSPO4 protein concentrations were tested: 35 pM, 350 pM, 3.5 nM and 35 nM (Table 2.10). MIN6 cell apoptosis was activated by Interleukin 1β (IL-1β), Tumor Necrosis Factor α (TNF-α) and Interferon γ (INF-γ) present in cytokine-containing treatment culture medium. The plate was incubated at 37°C for 20 h in a tissue culture incubator. 75 µl was removed from each well followed by addition of 25 µl of Caspase Glo substrate reconstituted in Caspase Glo buffer. The plate content was mixed on a plate shaker for at 100 g at room temperature for 1 min, which was followed by 1 h incubation at room temperature. The generated luminescent signal (excitation: 490nm, emission: 510-570 nm) was detected and analysed using Varitas luminometer (Turner Biosystems).

Table 2.9. Culture media used for the MIN6 cell apoptosis assay.

Medium	Composition
DMEM growth medium (Sigma)	25 mM Glucose, 10% Fetal Bovine Serum, 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol
2% serum DMEM (Sigma)	25 mM Glucose, 2% Fetal Bovine Serum, 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol
2% serum, cytokine-containing DMEM (Sigma)	25 mM Glucose, 2% Fetal Bovine Serum, 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol, 0.1 U/µl IL-1β, 2 U/µl TNF-α, 2 U/µl INF-γ

Table 2.10. Plate treatment map of the MIN6 cell apoptosis assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		No RSPO4	35 pM RSPO4	350 pM RSPO4	3.5 nM RSPO4	35 nM RSPO4	No RSPO4	35 pM RSPO4	350 pM RSPO4	3.5 nM RSPO4	35 nM RSPO4	
C												
D												
E												
F												
G												
H												
		2% serum DMEM					2% serum, cytokine-containing DMEM					

2.11 Measuring insulin secretion from MIN6 cells

MIN6 cells were detached from the culture flask by incubation with 0.05% Trypsin/0.02% EDTA solution (Sigma) at 37°C for 3 min. The cell number and viability were measured in the cell suspension using Celltess™ automatic cell counter (Invitrogen). 25000 cells were plated per one well of a 96-well cell culture plate and cultured in 200 µl of DMEM growth medium at 37°C for 24 h (5% CO_2, 95% air) in a tissue culture incubator. Table 2.11 presents details of culture media and buffers used for the MIN6 cell insulin secretion assay. The next day medium was replaced in each well with 200 µl of low glucose DMEM and the plate was incubated overnight (16 h) at 37°C in a tissue culture incubator. Following the overnight incubation, medium was removed and the plate wells were washed with 200 µl of low glucose Gey & Gey buffer. After the washing step, 200 µl of low glucose Gey & Gey buffer was added in wells followed by 2 h incubation at 37°C in a tissue culture incubator. The buffer was then removed, treatment solutions were added in wells according to a plate treatment map (Table 2.12) and the plate was incubated at 37°C for 1 h in a tissue culture incubator. A known insulin secretion potentiator phorbol myristate acetate (PMA) (Sigma) was used as a positive control of β-cell insulin secretion [199]. The plate was then centrifuged at 800 g for 3 min and 150 µl of the supernatant were transferred into a new plate for determination of insulin concentration. Insulin concentration was measured in samples using an insulin radioimmunoassay technique (section 2.12).

Table 2.11. Culture media and buffers used for the MIN6 cell insulin secretion assay.

Medium/Buffer	Composition
DMEM growth medium (Sigma)	25 mM Glucose, 10% FBS, 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol
Low glucose DMEM (Sigma)	5.5 mM Glucose, 10% FBS, 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 µM 2-mercaptoethanol
Low glucose Gey & Ge buffer (Sigma)	2 mM Glucose,111 mM NaCL, 5 mM KCL, 27 mM $NaHCO_3$, 1 mM $MgCL_2$ x 6 H_2O, 0.22 mM KH_2PO_4, 0.28 mM $MgSO_4$ x $7H_2O$, pH 7.4
High glucose Gey & Gey buffer (Sigma)	20 mM Glucose,111 mM NaCL, 5 mM KCL, 27 mM $NaHCO_3$, 1 mM $MgCL_2$ x 6 H_2O, 0.22 mM KH_2PO_4, 0.28 mM $MgSO_4$ x $7H_2O$, pH 7.4

Table 2.12. Plate treatment map of the MIN6 cell insulin secretion assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		No RSPO4	350 pM RSPO4	3.5 pM RSPO4	35 nM RSPO4	500 nM PMA	No RSPO4	350 pM RSPO4	3.5 pM RSPO4	35 nM RSPO4	500 nM PMA	
C												
D												
E												
F												
G												
H												
	Low glucose Gey & Gey (2 mM glucose)						High glucose Gey & Gey (20 mM glucose)					

2.12 Insulin radioimmunoassay

The effect of RSPO4 protein on insulin secretion from MIN6 cells was measured using a radioimmunoassay (RIA) method. RIA is a highly sensitive technique used to analyse low, nanomolar/picomolar, antigen concentrations in biological samples. In insulin RIA, insulin is radioactively labelled with iodine-125 (^{125}I) and bound to an anti-insulin mouse antibody. A limited and known amount of anti-insulin antibody is used. A sample to be analysed is then added, resulting in a

competitive reaction of the ^{125}I-labelled insulin (tracer) and the unlabelled insulin from the sample, with the anti-insulin antibody. As a result of the competition for the antibody, a certain amount of ^{125}I-insulin will remain unbound to the antibody, and the amount of unbound radioactive insulin is proportional to the amount of unlabelled insulin in the sample. Free insulin and insulin bound to the antibody are separated and radioactivity of the labelled insulin bound to the antibody is measured. A standard curve, constructed using known concentrations of insulin, allows determination of the amount of insulin in the experimental samples.

100 µl of 1 in 5 diluted samples or standard insulin solutions were mixed with 200 µl of the ^{125}I-insulin and anti-insulin antibody mixture, prepared in borate buffer (Table 2.13). Triplicates of insulin standards (0.04-10 ng/ml) and duplicates of samples were assayed. Tubes containing assayed samples and standards were incubated at 4°C for 48 h to establish equilibrium. Following the incubation, the antigen-antibody complexes were precipitated with 1 ml of the precipitation buffer and centrifuged (1300 g, 4°C, 15 min). The supernatant was discarded and radioactivity of the remaining pellet, expressed as counts per minute (cpm), was determined using 2470 Automatic Gamma Counter (PerkinElmer). A standard curve, generated by plotting cpm against standard insulin concentration, was used to quantify sample insulin concentration (ng/ml).

Table 2.13. Culture media and buffers used for the insulin radioimmunoassay.

Buffer	Composition
Borate buffer (Sigma)	133 mM Boric acid, 10 mM EDTA, 67.5 mM NaOH, 1 mg/ml Bovine Serum Albumin (BSA), pH 8.0
PBS buffer (Sigma)	137 mM NaCL, 2.7 mM KCL, 10 mM $Na_2HPO_4 * 2H_2O$, 2 mM KH_2PO_4, pH 7.4
Precipitation buffer (VWR)	1x PBS, 15% Polyethylene glycol, 1 mg/ml γ-globulin, 0.05% Tween-20

2.13 Statistical analysis

For comparing two groups, obtained results were analysed by un-paired Student's t-test with Welch's correction. For comparing three and more groups, one-way analysis of variances (ANOVA) with Tukey's (equal variances) or Dunnett's T3 (unequal variances) post hoc tests, or two-way ANOVA with Bonferroni's post hoc test were used. Group variances were compared using Bartlett's test for equal variances. Data were processed using Prism (GraphPad) or SPSS Statistics 23 (IBM) software. Values were expressed as means ± standard error of the means (SEM). Statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Chapter 3

Analysis of mouse pancreatic β-cell proliferation during pregnancy

Chapter 3

3.1 Introduction

During pregnancy, in response to the increased demand for insulin, the maternal islets adapt by increasing both the insulin secretory response and the β-cell mass. An increase in the maternal β-cell mass is achieved through upregulation of β-cell proliferation and islet volume during early and mid-stages of pregnancy [31, 32]. After parturition the β-cell mass rapidly reverts to non-pregnant levels, which is associated with a drop in β-cell proliferation and enhanced cell apoptosis.

Placental lactogens (lactogen-I and lactogen-II) and prolactin are involved in upregulation of β-cells during pregnancy and are central to the pregnancy adaptation mechanism [30]. Moreover, lactogenic hormones induce serotonin synthesis in islets, which is related to upregulation of β-cell proliferation [56]. Serotonin activates its GPCR receptor HTR2B on the plasma membrane, leading to augmentation of the β-cell proliferation rate. At late stages of pregnancy and post-partum, the pro-proliferative effect of lactogenic hormones is counteracted by glucocorticoids, triggering suppression of β-cell proliferation and activation of cell apoptosis [62]. The drop in maternal β-cell proliferation rate at the end of pregnancy in rats correlates with an increase in maternal plasma cortisol concentration [200]. Prolactin and placental lactogens exert their functions through JAK kinase associated prolactin receptor, located in the cell membrane, whereas the glucocorticoid receptor (GCR) upon activation undergoes translocation from the cytosol to the nucleus, where it acts as a transcription factor.

However, these β-cell adaptations to pregnancy and particularly their reversal post-partum are still poorly understood. It is not yet known whether there is a selective loss of proliferating “new” cells at late stages of pregnancy and post-partum.

The study described in this chapter aimed to characterise the changes in β-cell proliferation and mass during pregnancy and post-partum in the mouse. The

specific experimental objectives were: to measure pregnancy-dependent and postpartum changes in β-cell proliferation and size as well as to assess whether lactation affected post-partum changes in the β-cell mass.

3.2 Methods

CBA/Ca mice were given BrdU, which was incorporated into the DNA of proliferating cells, in their drinking water as a 1 mg/ml solution for 10 days (section 2.1.1). Mice were culled one day after finishing the treatment. Immunohistochemical staining of pancreatic sections labelled with BrdU was then prepared (section 2.2). The sections were then visualized using a fluorescent microscope and images were acquired at x200 magnification. For each islet image the number of BrdU labelled β-cells and the total number of β-cells were counted using ImageJ software. The cross-sectional area of individual β-cells was analysed on the same sections as those used for the proliferation analysis.

3.3 Results

3.3.1 Changes in pancreatic β-cell replication during pregnancy

β-cell proliferation in mice given a thymidine analogue BrdU for gestational days 8-17 (early, mid-pregnancy) was significantly higher than in non-pregnant and post-partum mice (Figures 3.1 and 3.2). Figure 3.1 presents micrographs of representative islets stained with fluorescence.

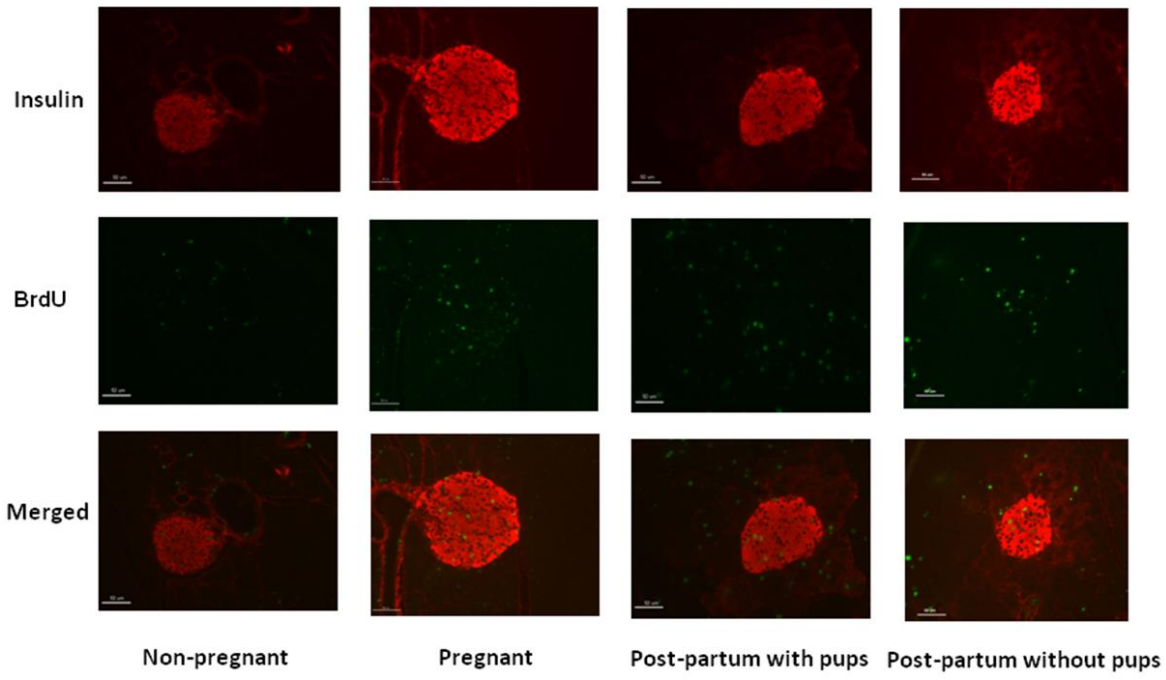

Figure 3.1. Fluorescent images of IHC stained islets. Pregnant mice (BrdU given for gestational days 8-17), post-partum mice left with pups (post-partum + pups) and post-partum mice left without pups (post-partum - pups) (BrdU given for days 1-10 postpartum). Non-pregnant control mice were given BrdU for 10 days.

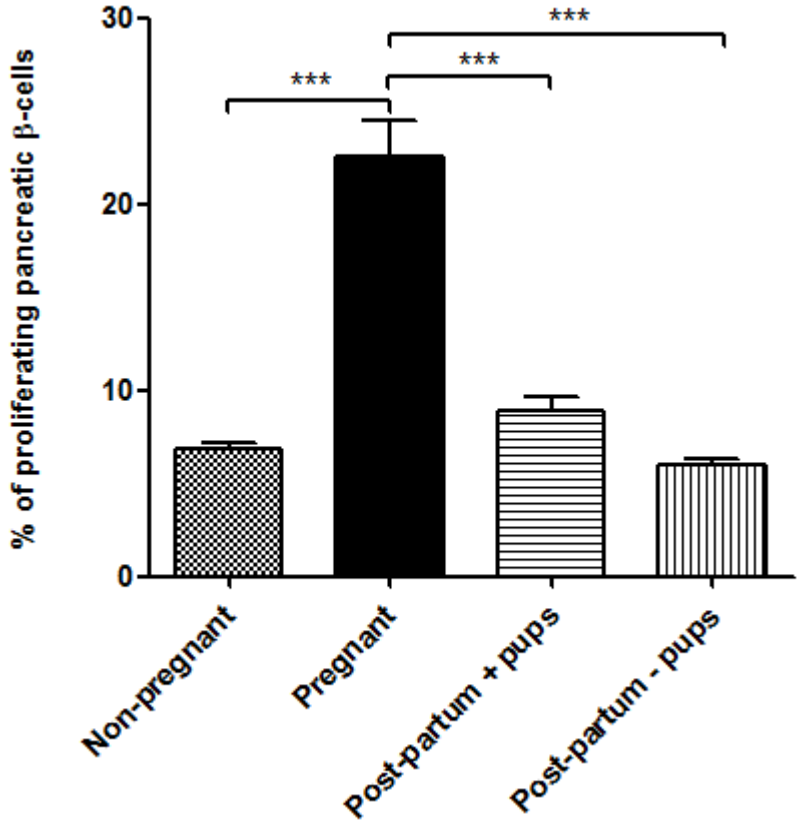

Figure 3.2. Analysis of the average β-cell proliferation rate. Pregnant mice (BrdU given for gestational days 8-17, n=4), post-partum mice left with pups (post-partum + pups) and post-partum mice left without pups (post-partum - pups) (BrdU given for days 1-10 postpartum, n=5). Non-pregnant control mice were given BrdU for 10 days (n=5). Values are expressed as means ± SEM; one-way ANOVA test * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

The β-cell size in pregnant mice was significantly increased compared to non-pregnant control mice and mice left without pups post-partum. Figure 3.3 presents the average β-cell size of analysed mouse groups.

The effects of lactation-related signals were investigated in post-partum mothers maintained for 10 days post-partum either with or without their litter. Female mice maintain lactation for as long as pups are present and fed regularly by the mother. Levels of lactation-related signals in mothers, such as prolactin, rapidly return to normal non-pregnant levels in the absence of their litter. Mice left with pups post-partum showed a small, statistically non-significant, increase in β-cell proliferation

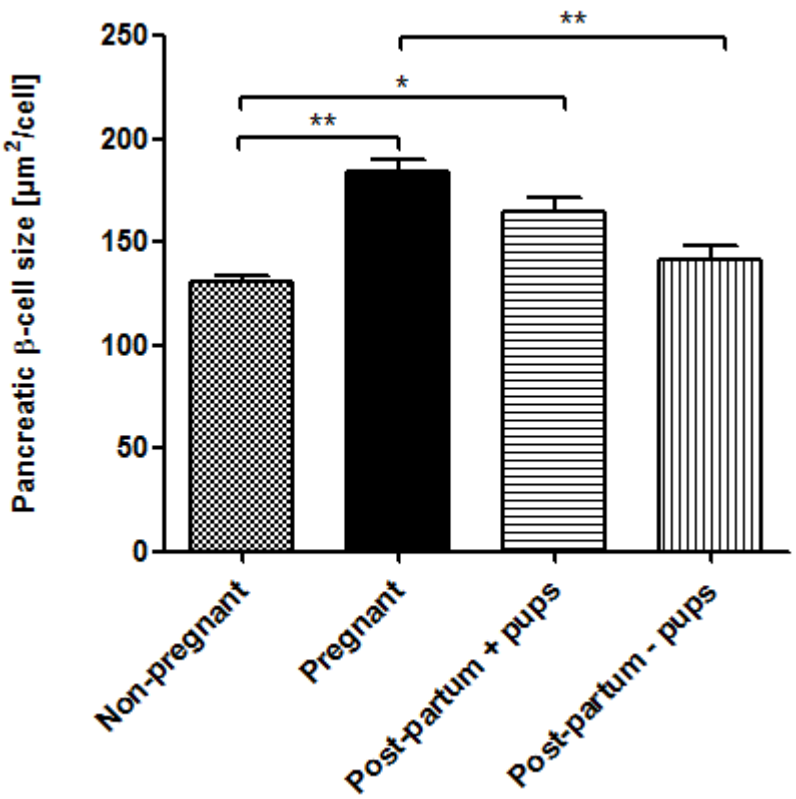

Figure 3.3. Analysis of average β-cell size. Pregnant mice (BrdU given for gestational days 8-17, n=4), post-partum mice left with pups (post-partum + pups) and post-partum mice left without pups (post-partum - pups) (BrdU given for days 1-10 postpartum, n=5). Non-pregnant control mice were given BrdU for 10 days (n=5). Values are expressed as means ± SEM; one-way ANOVA test * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

compared to both non-pregnant mice and mice kept without litter post-partum (Figure 3.2). In the absence of lactation, post-partum β-cell proliferation returned to the non-pregnant level. Interestingly, the β-cell size of post-partum mice maintained with pups was increased compared to the non-pregnant control group whereas there was no statistically significant difference in the size of β-cells of mice left without pups post-partum and the control (Figure 3.3).

3.3.2 Loss of β-cells post-partum

It is well known that β-cell mass increases temporarily during pregnancy through increased proliferation and hypertrophy but starts to drop back to non-pregnant levels after parturition. The main mechanisms involved in the post-partum reduction in the β-cell mass are decreased proliferation and increased apoptosis. Nevertheless, it is not clear which β-cells are lost through apoptosis post-partum, the newly generated or the older pre-existing β-cells.

In non-pregnant mice, the level of β-cell BrdU labelling was significantly higher immediately following BrdU treatment than in the case of samples taken from animals 10 days after the end of BrdU administration (6.9±0.6% vs 4.8±1.3%) (Figure 3.4). Therefore, in the non-pregnant mice, 31% of new β-cells, that proliferated over the initial BrdU labelling period of 10 days, were lost after the subsequent 10 days.

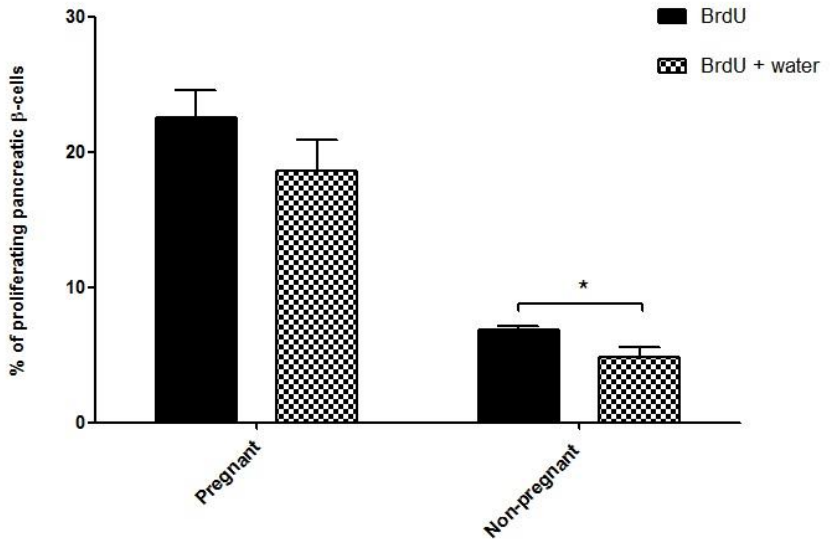

Figure 3.4. Analysis of the post-partum β-cell loss rate. Pregnant mice were orally administered BrdU for gestational days 8-17. After that period, mice were either culled (pregnant BrdU) or maintained for additional 10 days post-partum without BrdU treatment (pregnant BrdU + 10 days) before collection of the pancreas. Non-pregnant mice were given BrdU for 10 days and either culled (non-pregnant BrdU) or maintained for a further 10 days without BrdU before pancreas collection (non-pregnant BrdU + 10 days). Values are expressed as means ± SEM, n = 4-5; Student's t-test * $p<0.05$.

Interestingly, despite the increased β-cell apoptosis post-partum, loss of BrdU labelled β-cells in mice treated with BrdU for gestational days 8-17 and maintained for a further 10 days was small and not statistically significant (Figure 3.4). In mice administered BrdU from day 8 to 17 of pregnancy, 22.6±3.9% of β-cells were labelled positive for BrdU. When those mice were left for a further 10 days post-partum, the percentage of BrDU labelled β-cells dropped to 18.6±4.6%, corresponding to a non-significant 18.6% decrease in the number of β-cells.

3.4 Discussion

It is likely that pituitary prolactin plays a role in the regulation of β-cell mass during lactation, given the high physiological levels of prolactin post-partum and extensive literature linking prolactin to β-cell proliferation [29, 47]. The β-cell proliferation rate in lactating mice is an effect of an interplay between pro-proliferative lactation related signals and anti-proliferative glucocorticoids [64]. Glucocorticoids levels increase post-partum, compared to pregnant and non-pregnant mice, which activates apoptosis leading to the post-partum drop in the β-cell proliferation rate. However, other physiological signals such as HGF, produced by islet endothelial cells, may also be involved in this process as it is been shown that HGF stimulates β-cell proliferation during pregnancy [59]. In addition, those non-placental signals may be also responsible for prolonging the β-cell hypertrophy seen during pregnancy in lactating mice.

The data generated in this study show that pancreatic β-cells of pregnant mice (gestational day 8-17) had a higher proliferation rate than non-pregnant controls. It has been established that if the suckling stimulus of pups is removed post-partum the mother will stop lactating and plasma prolactin levels will drop to non-pregnant levels [201]. There was a significant drop in the β-cell proliferation rate immediately after parturition. In addition, the proliferation rate during lactation (mice kept with pups post-partum) was higher but not significantly different from the non-pregnant levels. Furthermore, ten days post-partum, a decrease in the β-cell size from its peak in pregnancy was observed. However, β-cells of the lactating mice were significantly larger than the control β-cells, which suggests involvement of the lactation-related signals, including prolactin and growth hormone, in maintaining β-cell hypertrophy and increased β-cell mass post-partum [202]. On the contrary, the β-cell size of the non-lactating mice was not significantly different from the non-pregnant controls. A potential role of non-placental signals in maintaining the augmented β-cell mass after parturition is supported by results of experiments on pseudopregnant mice. Pseudopregnant mice, generated through mating female mice with sterile males, have plasma

hormone levels mimicking those during pregnancy. Our team previously discovered that the pseudopregnant mice administered BrdU for days 4-12 of pseudopregnancy have significantly increased both the β-cell size and the proliferation rate, compared to the non-pregnant controls, but lower than in the pregnant mice administered BrdU for gestational days 4-12 [203].

Moreover, the importance of lactation for improved β-cell function is highlighted by studies in breastfeeding women with GDM, who have a reduced risk of developing T2DM compared to non-breastfeeding mothers with GDM [204].

Growth hormone comprises a link between placental and non-placental signals involved in adaptations to pregnancy. In humans placenta is the main source of circulating growth hormone during pregnancy, replacing the pituitary growth hormone production until shortly before parturition [205]. However, expression of rat placental growth hormone decreases during pregnancy but increases in the pituitary [206]. Growth hormone takes part in increasing insulin resistance and supply of nutrients for the fetus during pregnancy but *in vitro* it is not as effective as prolactin at stimulation of β-cell proliferation and insulin secretion [207-209]. In addition, growth hormone is a member of the prolactin gene family and can bind to the prolactin receptor [210].

Scaglia and colleagues reported 2.6 times higher than the control β-cell proliferation rate at the end of pregnancy and a return of the β-cell proliferation rate to the control level 10 days post-partum [38]. In addition, the β-cell size was significantly decreased 10 days postpartum compared to the non-pregnant control mice. As opposed to the Scaglia's results, in this study, the augmented β-cell size was observed 10 days post-partum. A different method of BrdU administration than in Scaglia's experiments was used in this study, constant for 10 days in drinking water. Scaglia used intraperitoneal BrdU injections at gestational day 20 and postpartum days 4, 8 and 10. In addition, Scaglia used rats in his experiments. Discrepancy between experimental protocols may explain differences in obtained results.

The mechanisms underlying β-cells mass reduction post-partum include reduction of the β-cell size, decreased cell proliferation and augmented apoptosis [38, 62]. Apoptosis selectively targets β-cells within the islet during that time [211]. However, it is not known if that reduction concerns the entire β-cell population or whether the β-cells proliferating during pregnancy are preferentially lost after parturition. Some researchers suggest that apoptosis may depend on the β-cell localization within the islet, with apoptotic β-cells localized preferentially, but not exclusively, at the periphery of the islets [38]. On the other hand, other experimental results support the role of mitosis as the vulnerability factor. Post-mitosis human β-cells are more vulnerable to apoptosis induced by islet amyloid polypeptide (IAPP) than non-dividing cells [211].

After termination of BrdU administration, BrdU labelling of β-cells drops with time proportionally to the rate of β-cell loss. Only proliferating cells are labelled with BrdU. Therefore, it is possible to measure the rate of proliferating β-cell loss by comparing the percentage of BrdU labelled cells just after finishing the BrdU administration with the percentage of BrdU labelled cells 10 days after finishing the BrdU treatment. In the pancreas collected from non-pregnant mice immediately after BrdU treatment there was BrdU labelling in 6.9±0.6% of β-cells, whilst in non-pregnant mice, whose BrdU treatment was followed by a further 10 days of water administration alone, that value dropped to 4.8±1.3% of BrdU labelling. Thus, we can say that in the non-pregnant mice significant 31% of β-cells, which proliferated over a period of 10 days, were lost over the subsequent 10 days. These results were compared to mice sacrificed immediately after being given BrdU from day 8-17 of pregnancy and mice administered BrdU from day 8-17 of pregnancy followed by water alone for 10 days post-partum. In principle, if β-cell mass returned to pre-pregnancy levels through a selective loss of the “new” β-cells formed during pregnancy, the post-partum drop in BrdU labelling of β-cells after 10 days would have been greater than the 31% seen after 10 days in the non-pregnant mice. In contrast, only 18.6% of the β-cells proliferating during gestational days 8-17 were lost within 10 days post-partum, which was not statistically significant. Based on

these observations, we can conclude that the return to the non-pregnant β-cell mass after pregnancy was not achieved through a selective loss of “new” β-cells formed during pregnancy and is regulated by different criteria.

Chapter 4

Analysis of the mouse placental GPCR ligand secretome and pancreatic islet GPCRome during pregnancy

Chapter 4

4.1 Introduction

During pregnancy, the mother undergoes major hormonal and metabolic changes, including increased β-cell mass, to ensure a continuous supply of nutrients to the fetus, as discussed in section 1.2. In mice, the highest rate of β-cell proliferation is observed on gestational days 13-15, dropping thereafter to reach almost control levels on day 18, just before parturition [32, 49]. Placental hormones and signalling through G protein-coupled receptors are crucial for those adaptive changes.

The placenta, in addition to its functions in mediating oxygen/carbon dioxide and nutrients/waste products exchange between the mother and the fetus, is also a major endocrine organ, which induces a wide range of changes in the maternal physiology and energy homeostasis in order to provide an ample supply of nutrients for itself and the fetus [68]. Placental lactogens and growth hormone increase maternal food intake, triggering insulin resistance, which leads to elevated levels of plasma glucose [40, 208, 212]. Furthermore, placental lactogens play a main role in activation of maternal β-cell mass expansion during pregnancy [56, 57, 213].

Serotonin mediates the pro-proliferative and insulinotropic action of lactogenic hormones in maternal β-cells through activation of its GPCR receptor HTR2B, Gαs protein-coupled, during mid-pregnancy stages [56, 58]. However, just before parturition HTR2B expression in β-cells is downregulated while expression of another serotoni receptor HTR1D, Gαi protein-coupled, is increased, which coincidences with a drop in β-cell proliferation. Moreover, disruption of serotonin-related GPCR signalling by knockdown of the brain serotonin receptor HTR2C in non-pregnant mice, increases the severity of T2DM [55]. These observations suggest that GPCR signalling plays a key part in the regulation of maternal β-cell mass expansion and function.

GPCR receptors comprise the largest and most diverse group of membrane receptors in Eukaryotes. Many drugs targeting GPCRs have excellent therapeutic benefits. Analogues of GLP1R (GLP-1 receptor), exenatide and liraglutide, are used in therapies against T2DM [214, 215]. Our group has recently established that 293 GPCR genes are expressed in human islets and most of them have unknown functions in islet physiology [170]. The placenta synthesizes a wide range of hormones and peptides, many of which are established ligands for GPCRs. Our knowledge of the islet GPCRome allows us to predict which of these placental ligands the islet cells may be able to respond to.

Therefore, in this study we will analyze expression of mouse known GPCR ligands in placentas and GPCRs in pancreatic islets at time of β-cell mass expansion (gestational day 12) and late pregnancy (gestational day 18), when β-cell mass expansion is terminated, to identify novel ligands/GPCRs, which may be involved in adaptive responses of islets to pregnancy. The prediction is that gene expression of GPCRs and/or corresponding GPCR ligands involved in β-cell mass upregulation during pregnancy will be higher on gestational day 12 than day 18. Analyzing expression of placental GPCR ligands and the corresponding islet GPCRs can further advance our understanding of β-cell adaptation to pregnancy.

4.2 Methods

Pancreatic islets (non-pregnant mice) or placentas and pancreatic islets (pregnant mice on gestational days 12 or 18) were isolated from female CD1 mice at 8 weeks of age, followed by isolation of placental and islet total RNA (sections 2.1.2, 2.3, 2.4). mRNAs isolated from mouse placentas on gestational day 12 and 18 as well as mRNAs isolated from islets of non-pregnant, gestational day 12 and 18 mice were converted into cDNAs, which were then used for quantitative RT-PCR gene expression studies (sections 2.5 and 2.6). Gene expression of 126 potential islet GPCR ligands (GPCR ligand secretome) and 342 islet GPCRs (GPCRome) was measured using the $2^{-\Delta\Delta Ct}$ method with GAPDH as a reference gene.

4.3 Results

4.3.1 Analysis of the mouse placenta GPCR ligand secretome during pregnancy

On gestational day 12, when β-cell proliferation is increased in mice, 79 out of 126 analysed known GPCR ligand genes were expressed at detectable levels in mouse placentas (Figure 4.1) [29]. On gestational day 12, 35 placental GPCR ligand genes were upregulated while 17 genes were downregulated compared to gestational day 18 expression levels.

On gestational day 18, characterised by decreased β-cell proliferation, expression of 62 placental GPCR ligand genes was detectable (Figure 4.2). On gestational day 18, 26 placental GPCR ligand genes were upregulated whereas 9 genes were downregulated compared to gestational day 12 expression levels. In addition, expression of 27 placental GPCR ligand genes did not change between gestational days 12 and 18.

Approximately half of the GPCR ligand genes expressed in the placentas at both time points have known functions in pancreatic β-cells (Figure 4.3).

Gene expression analysis of prolactin, placental lactogen-I and lactogen-II, known regulators of β-cell adaptations to pregnancy, in the placentas revealed that lactogen-II was the most highly expressed gene on both gestational day 12 and day 18 (Figure 4.4) [40]. The lactogenic hormones receptor (PRLR) belongs to the type I cytokine receptor family, activating Jak2 kinase, and does not belong to the GPCR family. In addition, lactogen-II expression dropped on gestational day 18 compared to day 12 expression levels. Maternal plasma concentration of lactogen-I increases during early pregnancy and peaks on gestational day 9 then drops sharply and remains constant until parturition in rodents [216, 217]. Maternal plasma concentration of lactogen-II is known to increase during mid-pregnancy,

peaking on days 12-13 of gestation and remaining high until parturition [216, 218]. Prolactin, a pituitary hormone, expression was not detectable in the placentas.

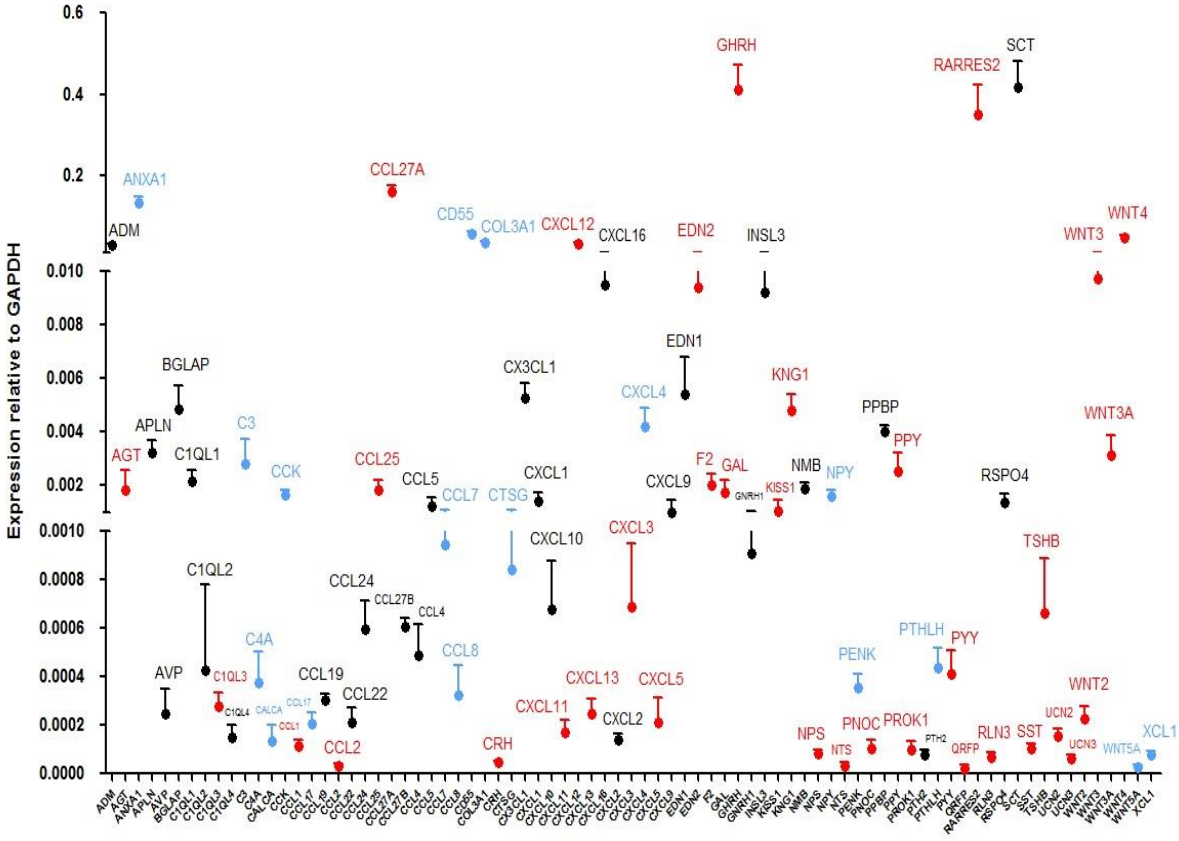

Figure 4.1. Gestational day 12 mouse placental GPCR ligand secretome. Red – genes upregulated on gestational day 12 vs day 18 expression levels, black – genes not differentially expressed on gestational day 12 vs day 18 expression levels, blue – genes downregulated on gestational day 12 vs day 18 expression levels. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 4-8; Student's t-test, red and blue $p<0.05$.

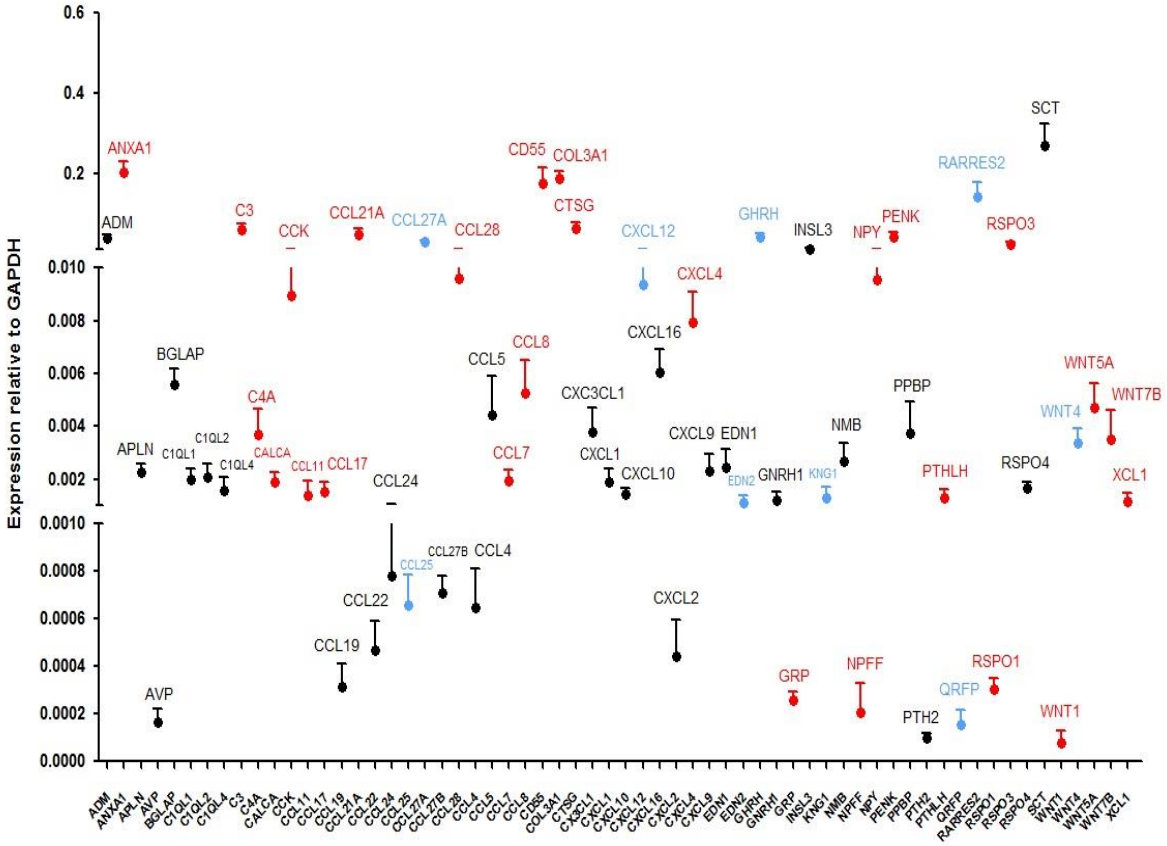

Figure 4.2. Gestational day 18 mouse placental GPCR ligand secretome. Red – genes upregulated on gestational day 18 vs day 12 expression levels, black – genes not differentially expressed on gestational day 18 vs day 12 expression levels, blue – genes downregulated on gestational day 18 vs day 12 expression levels. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 4-8; Student's t-test, red and blue $p<0.05$.

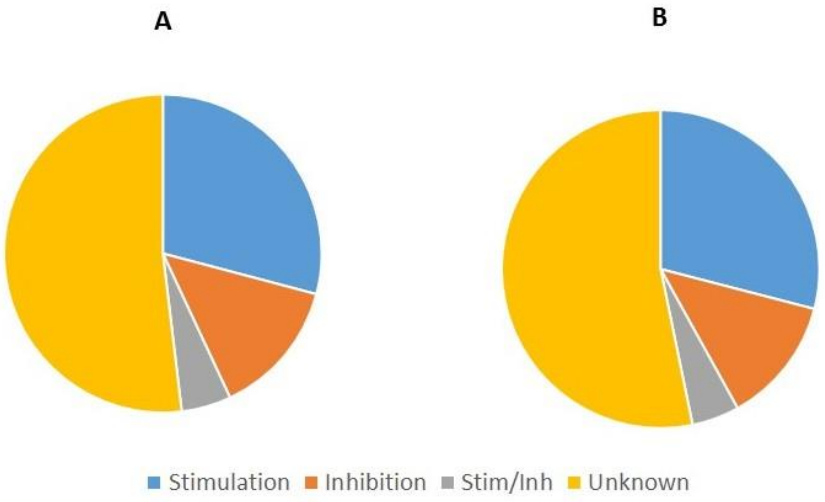

Figure 4.3. Effects of placental GPCR ligand genes expressed on gestational day 12 (A) and day 18 (B) on human and/or rodent β-cell insulin secretion and β-cell proliferation. Stimulation – stimulatory effects of ligands on β-cell insulin secretion and/or β-cell proliferation, Inhibition – inhibitory effects of ligands on β-cell insulin secretion and/or β-cell proliferation, Stim/Inh – stimulatory or inhibitory effects of ligands on β-cell insulin secretion and/or β-cell proliferation depending on experimental conditions or species, Unknown – unknown effects of ligands on β-cell insulin secretion and/or β-cell proliferation.

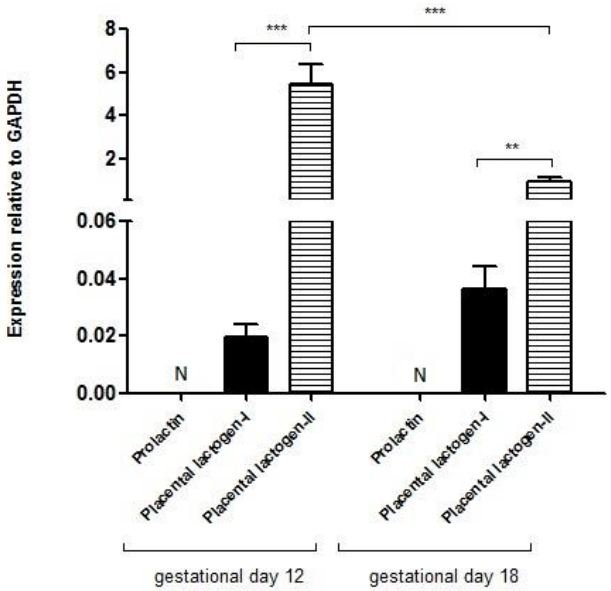

Figure 4.4. Analysis of lactogenic hormones gene expression in the mouse placentas on gestational days 12 and 18. N – not detectable/not quantifiable expression. Values are expressed as means ± SEM, n=5; two-way ANOVA test ** p<0.01, *** p<0.001.

4.3.2 Analysis of the mouse pancreatic islet GPCRome during pregnancy

In total, expression of 191 out of the 342 analysed GPCR genes was quantifiable in mouse pancreatic islets on gestational day 12 (Appendix III). 71 GPCR genes were differentially expressed in the islets on gestational day 12 compared to non-pregnant expression levels (Figure 4.5). 36% of the genes differentially expressed on day 12 were upregulated compared to non-pregnant expression levels. In addition, 15 of those GPCR genes were upregulated compared to both non-pregnant and gestational day 18 expression levels.

Gene expression of 195 GPCR genes was quantifiable in pancreatic islets on gestational day 18 (Appendix III). Moreover, 66 GPCR genes were differentially expressed in the islets compared to non-pregnant expression levels (Figure 4.6). 50% of the genes differentially expressed at that time point were upregulated compared to non-pregnant expression levels. In addition, 26 of those GPCR genes were upregulated compared to both non-pregnant and gestational day 12 expression levels.

Furthermore, on gestational day 12, there were 18 GPCR genes differentially expressed in the pancreatic islets compared to non-pregnant expression levels, with their corresponding ligands expressed in placentas (Figure 4.7). 38% of those GPCR genes were upregulated compared to non-pregnant expression levels.

There were 13 GPCR genes upregulated in the islets on gestational day 12 compared to non-pregnant and/or day 18 expression levels, whose ligands where also expressed in the placentas at that time point (Figures 4.9 and 4.10). 5 of those genes were chemokine or complement protein receptors (Figure 4.10).

In comparison, at the gestational day 18, there were 17 GPCR genes differentially expressed in the pancreatic islets compared to non-pregnant expression levels, with their corresponding ligands expressed in the placentas (Figure 4.8). 58% of

those GPCR genes were upregulated compared to non-pregnant expression levels.

PRLR, the lactogenic hormones receptor, gene expression in the islets was upregulated on both gestational days 12 and 18 compared to non-pregnant expression levels (Figure 4.11).

A literature search was conducted to determine what was already known about the effect of activation of β-cell GPCRs whose expression was differentially expressed in islets on gestational days 12 and 18 compared to non-pregnant levels. The assumption behind this analysis was that those receptors whose expression was upregulated in pregnancy are more likely to be involved in β-cell adaptive responses to pregnancy (Appendix IV).

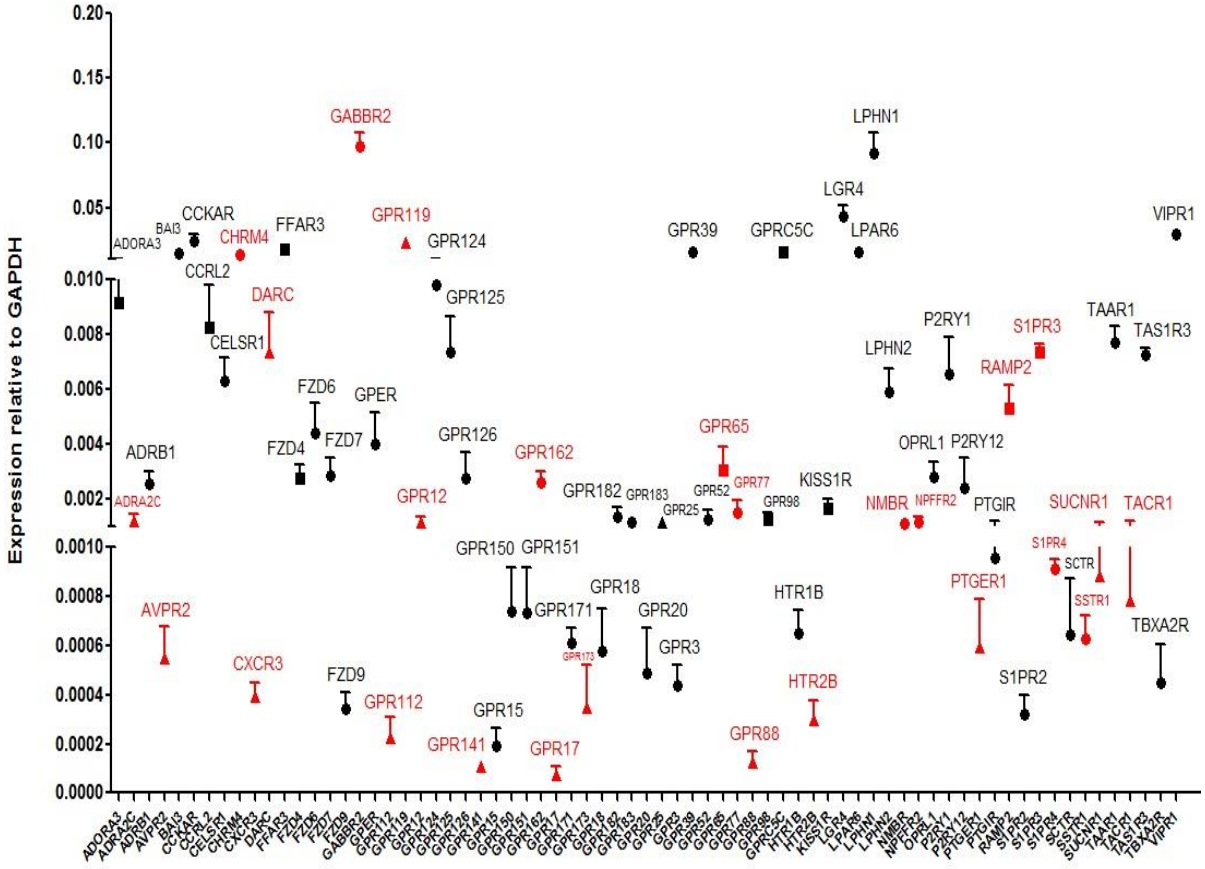

Figure 4.5. GPCR genes differentially expressed in mouse pancreatic islets on gestational day 12 compared to non-pregnant expression levels. Red – genes upregulated on gestational day 12 vs non-pregnant expression levels, black – genes downregulated on day 12 vs non-pregnant expression levels, triangle – genes upregulated on day 12 vs day 18 expression levels, circle – genes not differentially expressed on day 12 vs day 18 expression levels, square – genes downregulated on day 12 vs day 18 expression levels. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 3-8; one-way ANOVA test, all the genes $p<0.05$.

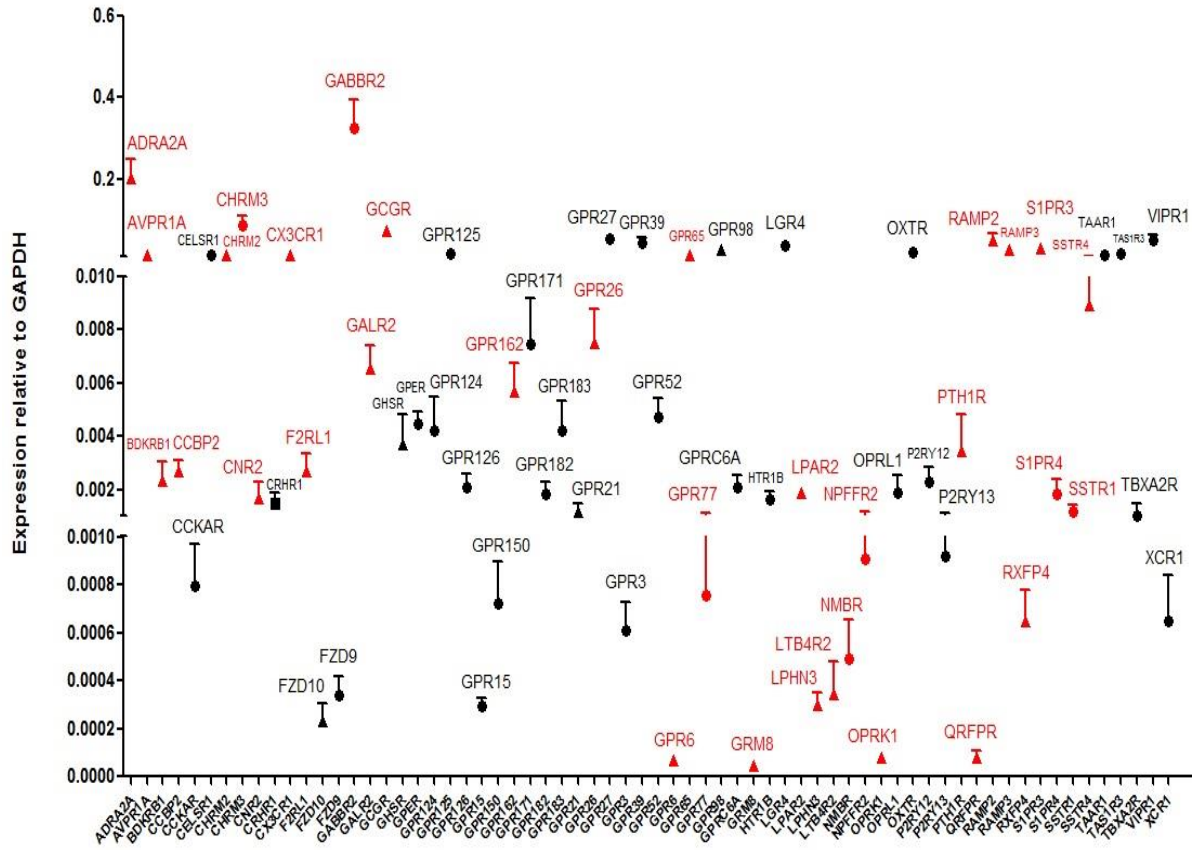

Figure 4.6. GPCR genes differentially expressed in mouse pancreatic islets on gestational day 18 compared to non-pregnant expression levels. Red – genes upregulated on gestational day 18 vs non-pregnant expression levels, black – genes downregulated on day 18 vs non-pregnant expression levels, triangle – genes upregulated on day 18 vs day 12 expression levels, circle – genes not differentially expressed on day 18 vs day 12 expression levels, square – genes downregulated on day 18 vs day 12 expression levels. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 3-8; one-way ANOVA test, all the genes $p<0.05$.

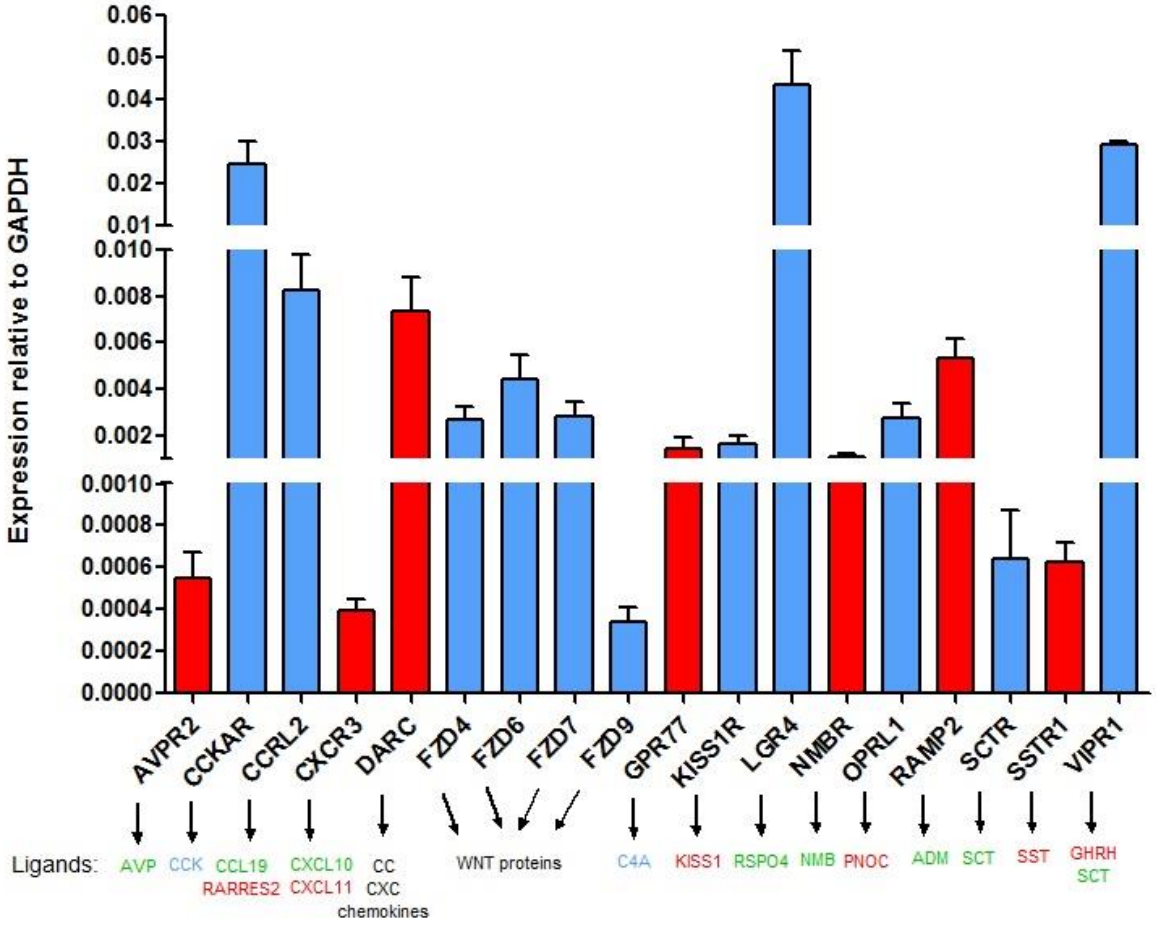

Figure 4.7. GPCR genes differentially expressed on gestational day 12 in mouse pancreatic islets, compared to non-pregnant expression levels, with their corresponding ligands expressed in placentas on gestational day 12. Little information on the specificity of FZD-WNT interaction is available due to the lack of purified and biologically active WNTs [219]. Therefore, WNT proteins labelled as a protein group were included in the figure. DARC is a non-specific receptor binding CC and CXC classes of chemokines. Red – genes upregulated on gestational day 12 vs non-pregnant (receptors) or day 18 (ligands) expression levels, blue – genes downregulated on day 12 vs non-pregnant (receptors) or day 18 (ligands) expression levels, green – placental GPCR ligand genes not differentially expressed on day 12 vs day 18 expression levels. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 3-8; Student's t-test (ligands) or one-way ANOVA test (GPCRs) were used, red and blue $p<0.05$.

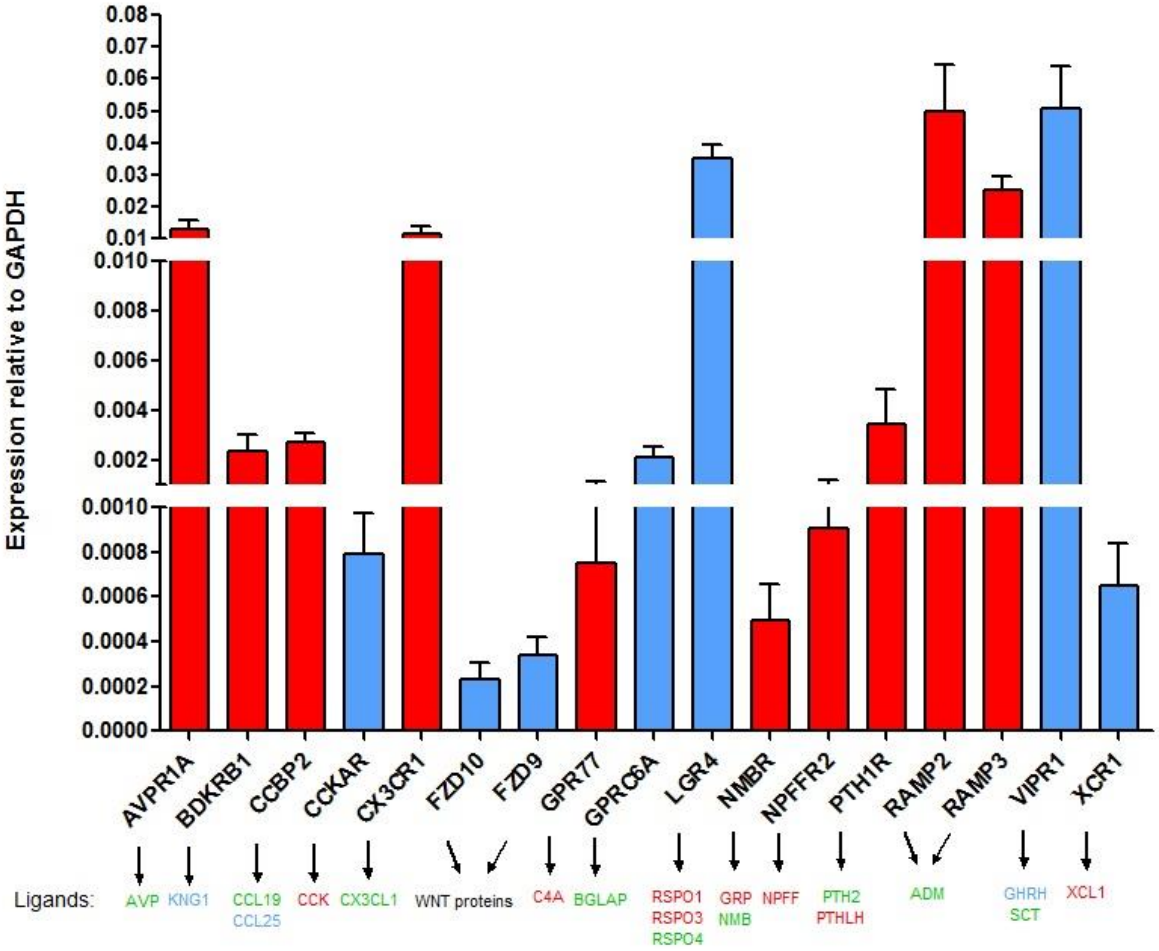

Figure 4.8. GPCR genes differentially expressed on gestational day 18 in mouse pancreatic islets, compared to non-pregnant expression levels, with their corresponding ligands expressed in placentas on gestational day 18. Little information on the specificity of FZD-WNT interaction is available due to the lack of purified and biologically active WNTs [219]. Therefore, WNT proteins labelled as a protein group were included in the figure. Red – genes upregulated on gestational day 18 vs non-pregnant (receptors) or day 12 (ligands) expression levels, blue – genes downregulated on day 18 vs non-pregnant (receptors) or day 12 (ligands) expression levels, green – placental GPCR ligand genes not differentially expressed on day 18 vs day 12 expression levels. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 3-8; Student's t-test (ligands) or one-way ANOVA test (GPCRs) were used, red and blue $p<0.05$.

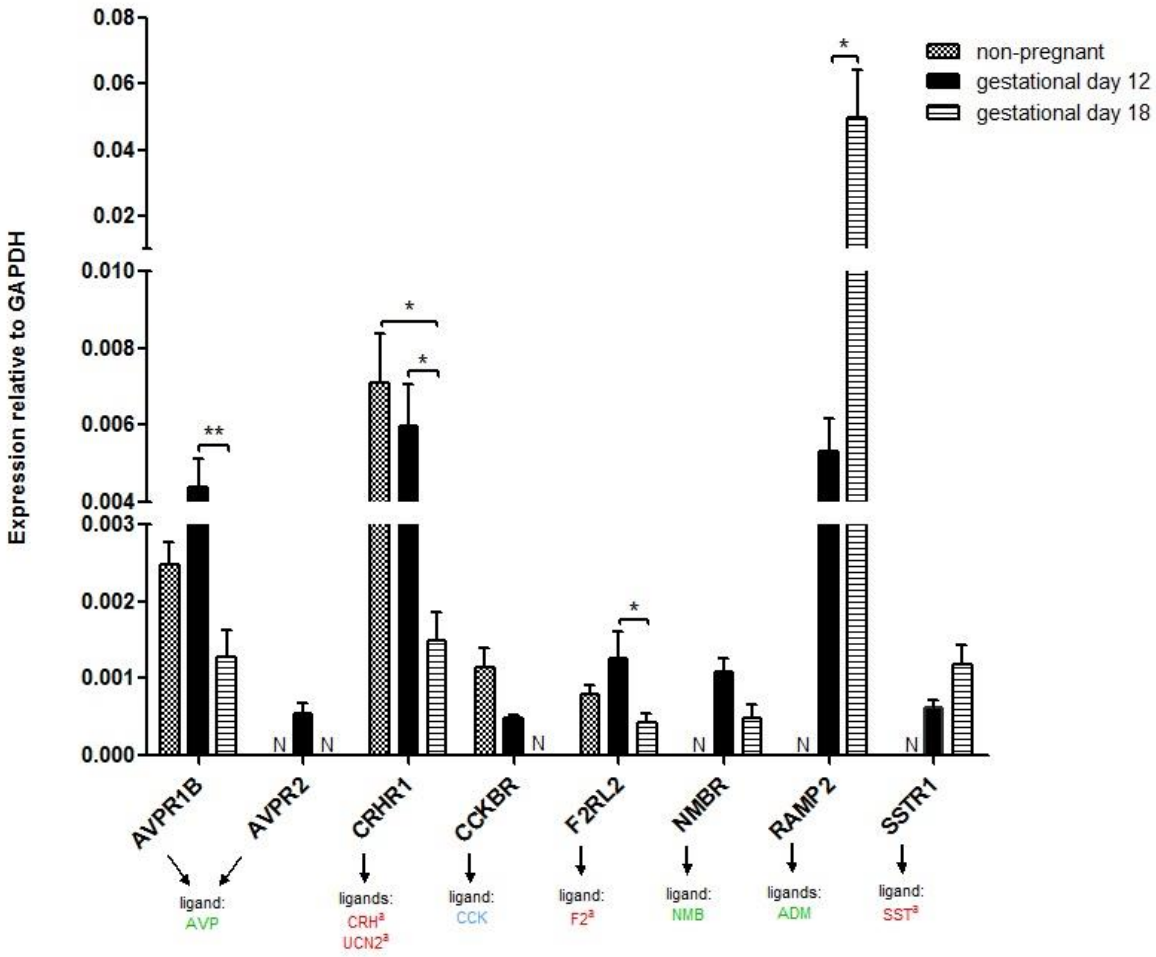

Figure 4.9. Mouse GPCR genes upregulated on gestational day 12 in pancreatic islets, compared to non-pregnant or/and gestational day 18 expression levels, with their corresponding ligands expressed in placentas on gestational day 12. Red – ligand genes upregulated on gestational day 12 vs day 18 expression levels, blue – ligand genes downregulated on day 12 vs day 18 expression levels, green - ligand genes not deferentially expressed on day 12 vs day 18 expression levels, N – not detectable/not quantifiable expression, [a] – ligand genes expressed in the placentas on gestational day 12 but not expressed on day 18. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 3-8; one-way ANOVA test * $p<0.05$, ** $p<0.01$.

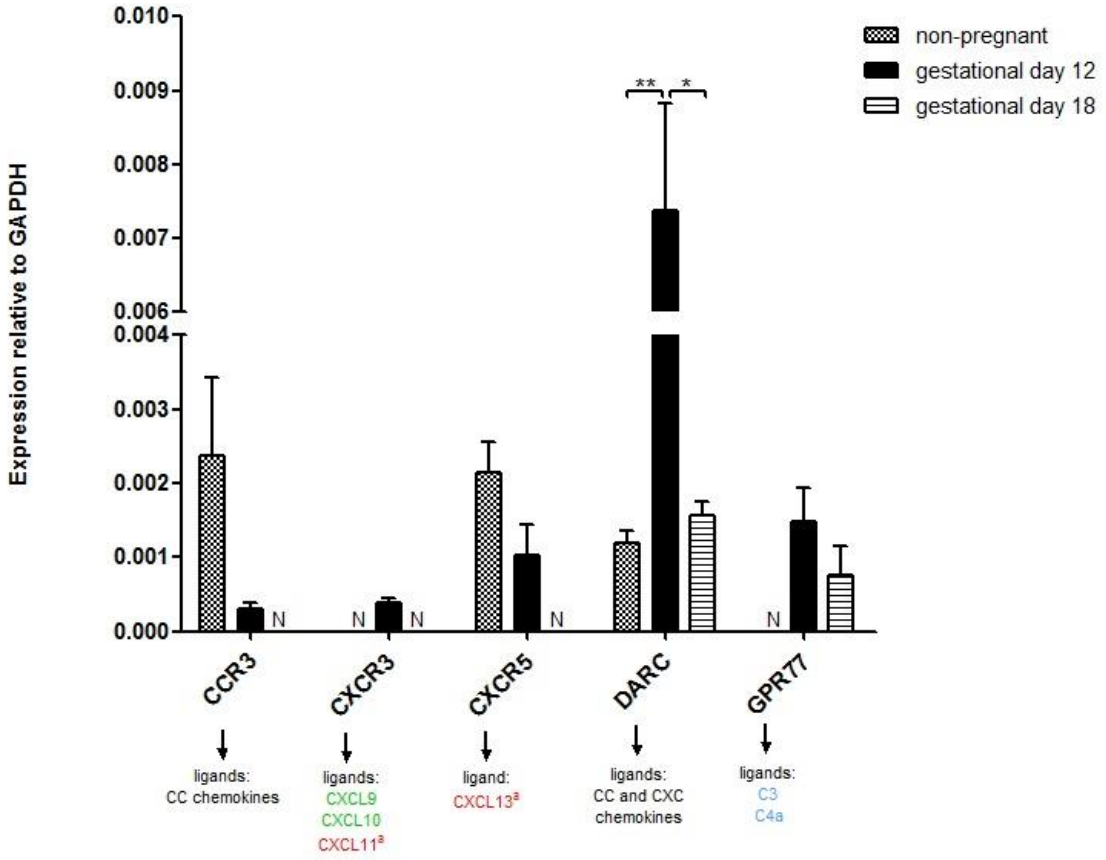

Figure 4.10. Mouse chemokine GPCR genes upregulated in pancreatic islets on gestational day 12, compared to non-pregnant or/and gestational day 18 expression levels, with their corresponding ligands expressed in placentas on gestational day 12. DARC is a non-specific receptor, which binds both CC and CXC classes of chemokines whereas CCR3 binds non-specifically CC chemokines. Red – ligand genes upregulated on gestational day 12 vs day 18 expression levels, blue – ligand genes downregulated on day 12 vs day 18 expression levels, green - ligand genes not deferentially expressed on day 12 vs day 18 expression levels, N – not detectable/not quantifiable expression, [a] – ligand genes expressed in the placentas on gestational day 12 but not expressed on day 18. Appendix II contains definitions of gene abbreviations. Values are expressed as means ± SEM, n = 3-8; one-way ANOVA test * $p<0.05$, ** $p<0.01$.

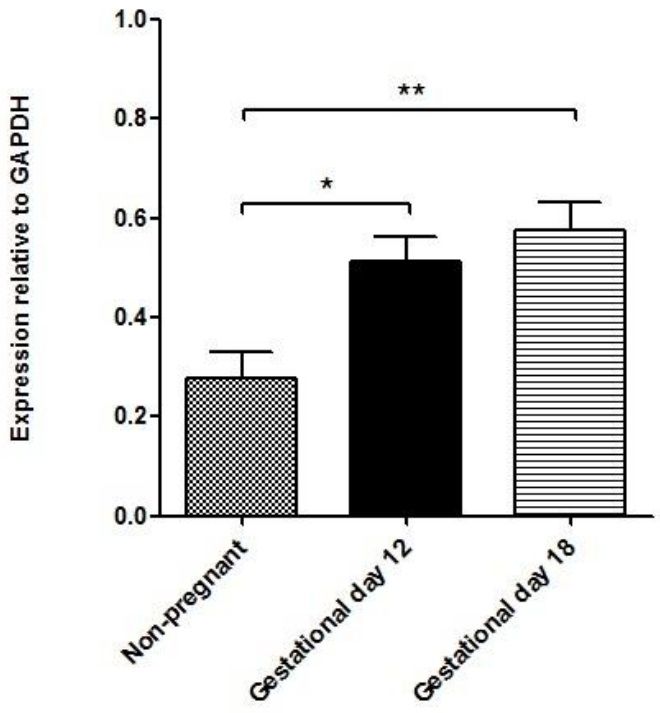

Figure 4.11. Gene expression of the mouse prolactin receptor in pancreatic islet during pregnancy. Values are expressed as means ± SEM, n = 3-8; one-way ANOVA test * $p<0.05$, ** $p<0.01$.

4.4 Discussion

The main aim of this part of the study was to identify likely placental GPCR ligands that regulate β-cell function during pregnancy. To do so, gene expression of known GPCR ligands and GPCRs was quantified in the placenta and pancreatic islets respectively. The criteria used for determining potentially implicated ligand/receptor genes included novelty of the gene function in β-cells, known pro-proliferative functions in other cell types, levels of gene expression as well as timing and a change of gene expression during pregnancy. In addition, the target positive regulator of β-cell function should have low probability of predictable serious side effects when used as a potential drug to improve β-cell function.

More GPCR ligand genes were expressed and upregulated in placentas on gestational day 12 than day 18. A high number of GPCR ligand genes expressed in placentas during pregnancy may be related to its endo- and autocrine functions. Many of those genes are involved in the maintenance of placenta function, the regulation of maternal physiology as well as regulation of fetal growth and development. For example, placental WNT proteins are involved in placentation and organogenesis, chemokines modulate the immune response and prevent rejection of the fetus by the mother's immune system while angiotensin regulates salt balance and maternal blood pressure during pregnancy [83, 220-222].

Differences in the number of total and upregulated ligands expressed in the placentas between day 12 and 18 may result from different physiological dynamics of mid- and late pregnancy. The placenta has a high metabolic demand, utilising 40% of oxygen delivered to the feto-placental unit [223]. Changes of the feto-placental metabolic demand depend on the pregnancy stage and embryogenesis processes. In mice, blastocyst implantation takes place on gestational day 4 while placental development completes on gestational day 16, corresponding to day 8-9 and week 13 of human pregnancy respectively [224]. Moreover, mouse gestational day 12 is characterized by dynamic changes in the developing genital system (gonad differentiation from a bi-potential to the sexually orientated state), presence

of 2 out of 7 rows of whiskers and heart chamber septation, while on day 18, thickening of the skin and whiskers elongation compared to day 17 as well as completion of nipple formation are observed [225-227]. Therefore, on day 12 the placenta is entering the period of maximum growth when the fetus is developing rapidly and the demands on the mother are greatest whereas on day 18 most of the fetal development is complete and the body of the mother is preparing to give birth and end the pregnancy.

In addition, approximately half of the GPCR ligand genes expressed on both gestational day 12 and 18 have known functions in pancreatic β-cells. Some of those genes may be involved in the regulation of β-cell mass and function during pregnancy. Given the number of GPCRs and their corresponding ligands that are expressed it is impossible to study all of them in detail. Thus it is important to consider which GPCR systems are most likely to play a role in the adaptation of the maternal islets to pregnancy based on the information available.

Expressed in the placenta on gestational day 12 growth hormone releasing hormone (GHRH), corticotrophin-releasing hormone (CRH) and somatostatin (SST) genes are elements of the placental hypothalamic-pituitary-adrenal (HPA) axis, responsible for the autocrine, paracrine and endocrine regulation of pregnancy physiology and stress responses [228, 229]. GH secretion from the pituitary gland, essential for pregnancy adaptations, is stimulated by GHRH and inhibited by somatostatin [230, 231]. Circulating GHRH concentrations in pregnant women are not significantly different from non-pregnant levels while GH concentrations are higher in pregnant compared to non-pregnant woman [232]. Furthermore, GH concentrations in cord blood are higher than in the maternal circulation which suggests that peripheral GHRH levels do not play an important role in maintaining circulating levels of GH during pregnancy. In the present study growth hormone releasing hormone receptor (GHRHR) expression in the islets did not change on gestational days 12 and 18 compared to non-pregnant levels. Although, GHRH gene expression was upregulated in the placentas on gestational day 12 compared to day 18, it has been shown that levels of plasma GHRH of

pregnant women do not differ from the non-pregnant levels [232]. Therefore GHRH was not chosen for further analysis. However, GHRHR antagonists stimulate proliferation and inhibit apoptosis of rat INS-1 β-cells [233].

Plasma concentrations of somatostatin in pregnant women rise from the eighth week until parturition and may play a part in relaxation of the gut [234]. In addition, expression of somatostatin receptor SSTR1 was upregulated in the islets on day 12 vs non-pregnant expression levels while somatostatin expression in placentas on day 12 was upregulated compared to day 18 levels. Somatostatin was not subjected to further analysis because it has an inhibitory effect on proliferation of various cell types and inhibits insulin secretion [235].

CRH plays a main role in upregulation of glucocorticoids release from adrenal glands during a stress response and is involved in placentation and labor onset [236]. Rising levels of CRH in the fetal brain as well as cortisol and corticotrophin (ACTH) in fetal circulation are observed as gestation progress, which contributes to maturation of organ systems [237]. Furthermore, CRH-mediated stimulation of maternal cortisol production results in increased glucose levels in maternal circulation, available for the fetus [238]. CRH promotes insulin secretion in isolated rodent pancreatic islets and upregulates both insulin secretion and cell proliferation in INS-1 β-cells [239]. It also induces glucagon secretion in rat and ovine islets [240]. The stimulatory effect of CRH on insulin secretion in mouse islets is potentiated by arginine vasopressin (AVP) [241]. AVPR1B and AVPR2 are receptors for arginine vasopressin. Dimerization of arginine vasopressin AVPR1B receptor and corticotrophin-releasing hormone CRHR1 receptor is necessary for synergistic CRH/AVP-dependent regulation of ACTH and insulin release [242]. Interestingly, both islet AVPR1B and CRHR1 genes as well as placental CRH gene were upregulated on gestational day 12 compared to day 18 expression levels whereas placental AVP gene expression did not change on days 12 and 18. AVP synergistically with CRH stimulates insulin secretion in mouse islets and ACTH release in the pituitary, the effect mediated by CRHR1-AVPR1B heterodimer [242]. Low amounts of AVP and CRH do not have a stimulatory effect

on ACTH secretion but application of both proteins results in significant secretion of ACTH [243]. Similarly, in mouse pancreatic islets AVP and CRH independently stimulate insulin secretion but the CRH effect is potentiated by AVP [241]. In addition, AVP stimulates glucagon secretion in mice as well as glucagon secretion and proliferation of In-R1-G9 α-cells through AVPR1B activation [244, 245]. The effect of AVPR2 receptor activation on the function of the endocrine pancreas is unknown. AVPR2 exerts its functions through a Gαs protein-adenylyl cyclase signaling pathway and mediates water reabsorption in the kidney [246]. Human plasma AVP concentration increase during pregnancy compared to non-pregnant controls, which is linked to increased thirst and water retention [247, 248]. Moreover, AVP is expressed and can be metabolically cleared in the placenta while regulating blood flow in the organ [249, 250]. In this study, AVPR2 gene expression in the islets was upregulated on gestational day 12 compared to non-pregnant and day 18 expression levels. AVP and CRH may be involved in upregulation of the β-cell function during pregnancy. However, these proteins were not used for further analysis because their stimulatory effects on β-cell proliferation (CRH) and insulin secretion (CRH and AVP) are already known.

Chemerin (RARRES2), fractalkine (CX3CL1) and osteocalcin (BGLAP) genes were expressed in the placenta on gestational day 12. Moreover, protein products of those genes have been shown to be present in serum of pregnant women [251-253].

Chemerin gene expression in the placentas on gestational day 12 was increased compared to day 18 expression levels. Chemerin is produced in adipose tissue and regulates adipocyte differentiation, lipolysis, glucose uptake and macrophage chemotaxis [254-256]. In addition, human chemerin is expressed at a relatively high level in the fetal and adult liver and omental fat but its mRNA expression is lower in the placenta [257]. There are three chemerin receptors: CMKLR1, CCRL2 and GPR1 [258-260]. In this study, a non-signalling decoy receptor CCRL2 was downregulated in the islets on gestational day 12 compared to non-pregnant controls. Gene expression of CMKLR1, the physiological receptor for chemerin, in

the islets did not change on gestational days 12 and 18 compared to non-pregnant mice while GPR1 expression was not detectable in the islets at both days 12 and 18.

Serum chemerin of pregnant women is positively associated with insulin resistance, BMI, and the plasma triglycerides concentration [251, 257]. In addition, serum chemerin is elevated in pregnant women and obese women with normal glucose tolerance compared to normal weight women with normal glucose tolerance [251]. A potential positive role of chemerin during human pregnancy is confirmed by analysis of circulating chemerin levels in women with GDM, which revealed that those women have decreased serum levels of chemerin compared to normal pregnancy levels [261]. Those observations suggest that, chemerin may act to reduce pregnancy-induced insulin resistance and prevent glucose intolerance in humans. Interestingly, chemerin was shown to increase insulin resistance in cultured human myocytes by disrupting insulin signalling and AKT phosphorylation [262]. Furthermore, chemerin improves glucose-dependent insulin secretion in mice but has counteracting modes of action in liver, adipose and skeletal muscle cells, which is manifested by inhibition of glucose production in the liver, impairment of insulin sensitivity in the muscle and improved insulin signaling in the adipose tissue [255]. Consequently, chemerin deficient mice and wild type controls have comparable total insulin sensitivity, demonstrated by intraperitoneal glucose tolerance test (IPGTT) [255]. In addition, islet area and morphology is not changed in chemerin knockout mice compared to the wild type control. Those observations suggest that chemerin plays different roles in the muscle, adipose tissue, and liver in the context of glucose homeostasis. Chemerin was not chosen for further analysis because the knockout of this gene in mice has no effect on β-cell mass [255].

Fractalkine (CX3CL1) is expressed in neurons, adipocytes, endothelial cells, hepatocytes vascular smooth muscle cells and the placenta. This molecule can exist in two forms: a soluble and a membrane-attached form. Fractalkine is involved in regulating chemotaxis of macrophages and can suppress inflammatory

activation of liver macrophages as well as pro-inflammatory cytokine release in the brain [263-265]. The fractalkine receptor CX3CR1 mediates fractalkine functions in pancreatic β-cells [266]. In this study, placental fractalkine gene expression did not change on days 12 and 18 whereas CX3CR1 expression did not change in islets on gestational day 12 but was upregulated on day 18 compared to non-pregnant expression levels. Circulating fractalkine levels are negatively associated with markers of insulin resistance and positively associated with markers of inflammation in pregnant women with GDM [252]. Moreover, human islets secrete fractalkine, which inhibits glucagon secretion and stimulates glucose-mediated insulin release [266, 267]. A stimulatory effect of fractalkine on insulin secretion has been also observed in mouse and rat islets [266, 267]. Human pancreatic β-cells express both fractalkine and its receptor. In addition, fractalkine has an anti-apoptotic effect in human β-cells [267]. There are species specific differences in the fractalkine mechanism of action as it has been reported that this protein has no effect on rat β-cell apoptosis [267]. Circulating fractalkine is positively associated with markers of inflammation in pregnant women with GDM and therefore it was not chosen for further analysis [252].

Post-translationally carboxylated osteocalcin (cOC) is one of the main organic components of the bone matrix secreted by osteoblasts and odontoblasts [268]. This protein is responsible for binding of calcium and hydroxyapetite, the mineral components of the bone. The circulating under-carboxylated osteocalcin (ucOC) has a lower affinity for the bone and plays a role in the regulation of energy metabolism. Osteocalcin exerts its functions in β-cells through activation of GPRC6A receptor [269, 270]. In this study, expression of the osteocalcin gene (BGLAP) in the placentas did not change on gestational day 12 compared to day 18. In addition, GPRC6A expression did not change on gestational day 12 but was downregulated on day 18 compared to non-pregnant expression levels. Some researchers reported that total circulating osteocalcin is increased in women with GDM compared to healthy pregnant women whilst others have not found any difference between women with and without GDM [253, 271]. On the other hand, decreased serum osteocalcin is observed in men with T2DM compared with non-

diabetic subjects [272]. Women who develop diabetes postpartum have lower serum levels of under-carboxylated osteocalcin [273]. Furthermore, human serum ucOC levels correlate with insulin resistance during pregnancy [271]. Orally administered ucOC indirectly improves glucose tolerance and basal insulin secretion in mice through stimulation of GLP-1 secretion [274]. In addition, ucOC can stimulate insulin secretion by upregulation of adiponectin release in mice [275, 276]. Intraperitoneally injected osteocalcin can also directly stimulate insulin secretion [270]. Moreover, ucOC increases β-cell proliferation and islet β-cell area [269, 274]. Interestingly, there is a link between fractalkine and osteocalcin. Both fractalkine and osteocalcin have two forms, a soluble and a plasma membrane-(fractalkine) or bone matrix-bound (cOC) forms. Furthermore, both proteins play important roles in bone physiology. Mouse fractalkine produced in the bone is implicated in osteoclast recruitment and osteoclastogenesis in mice [277]. Osteocalcin was not chosen for further analysis because its functions in β-cells have already been established.

Similarly to the placental ligand expression analysis, an expression profile of differentially expressed islet GPCR genes on gestational day 12 compared to non-pregnant levels was different from the day 18 profile. Although a comparable number of GPCR genes, whose ligands were expressed in the placentas, was differentially expressed on day 12 and 18 vs non-pregnant (18 and 17 genes respectively), more receptor genes were upregulated on day 18 than 12 vs non-pregnant expression levels (10 and 7 genes respectively). The observed differences between the GPCR gene expression profiles of these time points may be due to a different physiological dynamics of mid- and late pregnancy.

The most interesting islet GPCR receptor genes in the context of potential involvement in the stimulation of β-cells mass expansion during pregnancy are genes with expression profiles mimicking islet prolactin receptor expression, a positive β-cell regulator, and whose ligands are expressed in the placentas on gestational day 12. Receptor genes upregulated on gestational day 12 vs non-pregnant levels include AVPR2, NMBR, RAMP2 and SSTR1. In addition, genes

which are upregulated on day 12 vs either non-pregnant (AVPR2, NMBR, RAMP2) or day 18 (AVPR1B, CRHR1, CCKBR, F2RL2) expression levels may also have a positive effect on β-cell function during pregnancy. Interestingly, the only islet GPCR gene upregulated on day 12 compared to non-pregnant expression levels, whose placental ligand expression was upregulated at that time point vs gestational day 18 levels, was a somatostatin receptor SSTR1. Somatostatin, the SSTR1 ligand, inhibits proliferation of various cell types and inhibits insulin secretion [235].

NMB has been detected in the central nervous system, placenta, pituitary gland, pancreas, gastrointestinal tissues, and several types of cancer cells [278]. NMB is involved in smooth muscle contraction, lowers body temperature acting on the brain and increases plasma glucose [279-281]. NMB by NMBR receptor activation in the myometrium initiates the onset of labour in pregnant mice [282, 283]. Moreover, NMB stimulates insulin secretion and has no effect on glucagon and somatostatin release in the canine pancreas whereas in the rat pancreas this protein has no effect on insulin and a stimulatory effect on glucagon secretion [284, 285]. NMB expression in mouse pituitary corticotrophs, secreting NMB into the systemic circulation, is upregulated by administration of CRH and suppressed by treatment with a glucocorticosteroid dexamethasone [286]. In addition, a pro-proliferative effect of NMBR receptor activation by NMB has been observed in rat osteoblasts [287]. Although, NMBR gene expression was upregulated in the islets on day 12 compared to non-pregnant levels and NMB gene expression was detectable in the placentas on day 12, the NMB/NMBR system was not chosen for further analysis because of species-dependent differences in its activation effects on insulin secretion and the established role in the onset of labour [282-285].

CCKBR receptor binds cholecystokinin (CCK) and gastrin (GAST) with similar affinity [288]. CCK is a satiety reducing factor in most mammals including humans [289]. Serum CCK levels are decreased in pregnant women with severe vomiting syndrome (hyperemesis gravidarum, HG) compared to normal pregnancy controls [290]. Furthermore, CCKBR-deficient mice are characterized by obesity, insulin

resistance and impaired insulin secretion [291]. CCK also stimulates somatostatin secretion in rat RIN-14B ∆-cell line, expressing CCKBR [292]. Both CCK and GAST upregulate insulin secretion in rodents and humans [115, 293]. Treatment of mouse and human pancreatic islets with CCK promotes β-cell proliferation whereas gastrin induces β-cell neogenesis in rats after 90% pancreatectomy [294, 295]. The CCK/CCKBR system was not chosen for further analysis because CCK gene expression in the placentas was downregulated on day 12 compared to day 18 expression levels and CCKBR gene expression in the islets was not changed on day 12 compared to non-pregnant levels.

A heterodimer of RAMP2 and calcitonin receptor-like receptor (CRLR) forms AM1 receptor. The main endogenous ligand of AM1 is adrenomedullin (ADM) but amylin and calcitonin can also bind AM1 with weaker potency. ADM is expressed in a wide range of organs including the placenta, brain, smooth muscles endothelial cells and testis [296]. In this study, ADM gene expression in the placentas on gestational day 12 did not differ from day 18 expression levels whereas RAMP2 gene expression in the islets was upregulated on day 12 compared to non-pregnant levels. During pregnancy vascular resistance and mean arterial pressure are reduced, whereas cardiac output, heart rate, and blood volume are elevated [297]. As a result, the placental vasculature remains in a state of near-maximal dilatation. ADM exerts natriuretic action on kidneys and peripheral vasculature to control fluid and electrolyte homeostasis and antagonizes angiotensin II secretion [298]. There is a three- to five-fold increase in ADM plasma levels of pregnant women in gestational week 8 compared to non-pregnant levels, which is maintained until parturition [299]. ADM plasma levels are also increased in non-pregnant patients with hypertension compared to normotensive controls [300]. In addition, a hypothetical role of ADM in the cardiovascular adaptation to pregnancy is supported by the evidence that rat plasma ADM concentrations increase progressively from early pregnancy to term and drop post-partum [301]. ADM is a potent vasodilator and plays a role in uterine receptivity, implantation and vascular remodelling of spiral arteries during pregnancy [302, 303]. Moreover, placental ADM is involved in the regulation of

placental vascularization [304]. ADM administration in rats inhibits insulin secretion but has no effect on glucagon secretion [305, 306]. Furthermore, ADM promotes proliferation of human colorectal cancer (CRC) cells and osteoblasts [307, 308]. The role of ADM in reducing blood pressure during pregnancy suggests that ADM in β-cells may act as a blood pressure sensor. Patients with hypertension are characterised by peripheral insulin resistance and hyperinsulinemia [309, 310]. Interestingly, treatment with some antihypertensive drugs, including thiazides, has been linked to insulin resistance and T2DM in humans [311, 312]. Conversely, improved peripheral blood flow to skeletal muscles facilitates glucose disposal to the tissues and α-blockers, which promote peripheral vasodilation, may improve glucose uptake and insulin sensitivity [313]. ADM-mediated downregulation of insulin secretion may counterbalance the vasodilation-mediated effect on plasma glucose and insulin sensitivity. ADM was not chosen for further analysis because of its inhibitory effect on insulin secretion [305, 306].

Thrombin, encoded by F2 gene, also known as activated blood-coagulation factor II, is one of the elements of the coagulation cascade, whose activation ultimately results in the stemming of blood loss. In addition, thrombin and its receptors have been implicated in placentation and myometrium contractility during labor in humans [314, 315]. Human plasma thrombin levels increase during early pregnancy compared to non-pregnant levels and remain stable throughout later stages of gestation [316]. Thrombin exerts its functions through activation of F2RL1, F2RL2, F2RL3 and F2R receptors. In the present study, thrombin gene expression in the placentas was upregulated on gestational day 12 compared to day 18 expression levels while F2RL2 gene expression in the islets did not change on day 12 compared to non-pregnant levels. A cryptic domain of F2RL2 (protease-activated receptor-3, PAR3) is cleaved by thrombin, generating a 6-amino acid sequence that acts as a “tethered ligand” to initiate the receptor or other thrombin receptors activation [317]. F2RL2 expression is associated with glycated haemoglobin in humans while thrombin treatment and F2RL2 activation result in potentiated insulin secretion in human β-cells and mouse islets [318]. Furthermore, hyperlipidemia, hyperglycemia and hyperinsulinemia have been

shown to enhance the coagulation activity and increase levels of circulating coagulation markers, including thrombin [319, 320]. A mechanism of thrombin action in T2DM has been proposed in which elevated thrombin leads to F2RL2 activation and subsequent PLC stimulation, resulting in an increase in intracellular Ca^{2+} levels and potentiation of insulin release [318]. Interestingly, F2RL1 activation results in suppression of insulin secretion in mice [107]. Furthermore, a “tethered ligand” generated during F2RL2 activation can also activate F2R and F2RL1, which highlights the complexity of the thrombin-mediated regulation of insulin secretion [317]. Although, it is not known if thrombin affects β-cell proliferation, a pro-proliferative effect of thrombin has been observed in human vascular endothelial cells [321]. Thrombin was not chosen for further analysis because it has either stimulatory or inhibitory effects on insulin secretion depending on the receptor involved in mediating its effects in β-cells [318].

Another group of islet GPCR receptors, upregulated on gestational day 12 compared to non-pregnant or/and day 18 expression levels, is the chemokine receptors group, consisting of CCR3, CXCR3, CXCR5, DARC, GPR77. Chemokines are implicated in placentation, protection against infections and confer the mother’s immune tolerance of the fetus [83, 322]. A non-specific CC chemokine receptor CCR3 is involved in triggering β-cell apoptosis and has been shown to be downregulated in mouse RIN-5AH β-cells by interleukin-1β (IL-1β) [323]. CXCR3 and its ligand CXCL10 suppress β-cell proliferation in mice whereas CXCL10 neutralization with a specific antibody prevents occurrence of diabetes in mice [324]. The CXCR3/CXCL10 system is involved in pathogenesis of T1DM in mice [325]. Furthermore, CXCL10 plasma levels are elevated in type 1 diabetic patients compared with healthy controls [326]. CXCL13, a CXCR5 receptor ligand, mediates a pro-proliferative and anti-apoptotic effect of GLP-1 in type 2 diabetic mice [327]. GPR77 binds complement system factors C3a, C4a and C5a, whose plasma levels are increased in patients suffering from T1DM compared to normal controls [328]. The β-cell function of a non-signaling DARC receptor is unknown. Chemokines were not chosen for further analysis because of their negative effects in β-cells and involvement in pathogenesis of T1DM.

Synthesised locally in β-cells serotonin is a known mediator of the pro-proliferative and insulinotropic action of lactogenic hormones in maternal β-cells during pregnancy [56, 58]. Because serotonin is a tryptophan deriverative, it is not possible to measure serotonin levels using quantitative RT-PCR. Similarly to the results obtained by Kim and colleagues, in this study, elevated expression of the serotonin receptor HTR2B was observed on gestational day 12 compared to both non-pregnant and day 18 levels [56]. In addition, only trace, non-quantifiable expression of HTR1D receptor was detected in the islets on days 12 and 18. Kim observed elevated expression of HTR1D on gestational day 17, compared to day 12 levels in C57bl mice which may be related to differences in timing and the methodology of his RT-PCR analysis (glucuronidase used as a reference gene) as well as a different mouse strain used.

R-spondins (RSPO1, RSPO2, RSPO3, RSPO4) potentiate WNT/β-catenin signalling, which plays an important role in cell proliferation and embryogenesis [158, 178, 329]. Mutations in the human RSPO4 gene are associated with inherited anonychia, manifested by the absence of fingernails and toenails [168, 169, 330]. In this study, RSPO4 was the only R-spondin gene whose expression was detectable in the placentas on gestational day 12 and its expression at that time point was not significantly different from the day 18 expression levels. In addition, the R-spondins receptor LGR4 was expressed in the islets at relatively high levels on both gestational days 12 and 18 although its expression at both time points was lower than non-pregnant levels. High expression levels of LGR4 in mouse pancreatic islets have been reported during pregnancy but its role in β-cell adaptation to pregnancy have not been investigated [331]. Moreover, the RSPO1 protein stimulates insulin secretion, cell proliferation and suppresses cytokine induced apoptosis in MIN6 β-cells [162]. Therefore, RSPO4 was chosen for further analysis and functional studies in β-cells.

Most of the GPCR genes differentially expressed on gestational day 12 play important roles in islet physiology and pregnancy adaptations. Some ligand and

receptor genes have pro-proliferative effects in β-cells but have not been implicated in β-cell expansion during pregnancy (CCK, CRH, CXCL13, GHRH, ucOC). Others have unknown effects on β-cell proliferation but pro-proliferative effects in other cell types (ADM, AVP, F2, NMB). Chemerin has no effect on β-cell proliferation. In addition, some of the analysed genes have negative effects on β-cell and/or other cell types proliferation (SST, CXCL10). Fractalkine stimulates or suppresses cell proliferation depending on the cell type [332, 333]. A further functional analysis of GPCR ligands and their receptors during pregnancy, which have known effects on β-cells, will help to broaden our understanding of β-cell adaptive mechanisms during pregnancy.

Chapter 5

A potential role for R-spondin 4 in β-cell adaptations to pregnancy

Chapter 5

5.1 Introduction

Analysis of placental GPCR ligand secretome and islet GPCRome revealed that there were 18 islet GPCR genes differentially expressed on gestational day 12 compared to non-pregnant expression levels, whose ligands were expressed in the placentas on day 12 (section 4.3.2, Figure 4.7). Within that group of GPCR genes I decided to further analyse β-cell function of the R-spondin 4 (RSPO4) protein because a literature search suggested that this protein may have a stimulatory effect on β-cells. The RSPO1 protein, a member of the same protein family as RSPO4, has been shown to exert insulinotropic and pro-proliferative effects in MIN6 cells [162].

R-spondins belong to a superfamily of thrombospondin type 1 repeat (TSR-1)-containing proteins. R-spondin 1 (roof plate specific spondin 1, RSPO1) was first discovered in the neural tube of developing mouse embryos [156]. The R-spondin protein family consists of RSPO1, RSPO2, RSPO3 and RSPO4, which share 60% sequence homology in vertebrates [146]. R-spondins potentiate WNT/β-catenin signalling, that plays a key role in cell proliferation, embryogenesis and stem cell maintenance [158, 178, 329]. In addition, R-spondins are involved in sex determination during embryogenesis, differentiation and placenta vasculature development [160, 164, 167]. Mutations in the human RSPO4 gene are implicated in inherited anonychia, a mild disorder characterised by the absence of fingernails and toenails [168, 169, 330]. R-spondins exert their functions through activation of leucine-rich repeat containing GPCRs: LGR4, LGR5 and LGR6.

The RSPO1 protein stimulates insulin secretion, cell proliferation and suppresses cytokine-induced apoptosis in mouse MIN6 cells [162]. Interestingly, RSPO1 is a negative regulator of mouse pancreatic β-cell neogenesis [163]. Furthermore, RSPO1 and RSPO3 genes are expressed in rat hypothalamic neurons where their expression is upregulated by insulin [171]. Injection of RSPO1 and RSPO3

proteins into the rat hypothalamus brain area results in inhibition of food intake [171].

Positive effects of RSPO1 and RSPO3 on β-cells and glucose homeostasis suggest that RSPO4, acting through the same receptors as RSPO1 and RSPO3, may play a stimulatory role in β-cell proliferation and secretory functions.

For a placental ligand to be involved in maternal β-cell adaptive responses to pregnancy we would predict high levels of expression of the ligand mRNA at the appropriate time point during gestation (early to mid-gestation) rather than late gestation, as well as maintained or increased expression of the β-cell receptor for the ligand at the appropriate phase of pregnancy. Moreover, we would anticipate increased concentration of the placenta-derived ligand in the maternal circulation, stimulatory effects of the ligand on β-cell proliferation and secretory responses as opposed to the inhibitory effect on β-cell apoptosis. The aim of the experiments described in this chapter was to determine whether RSPO4 fulfilled those criteria.

5.2 Methods

Pancreatic islets (non-pregnant mice) or placentas and pancreatic islets (pregnant mice on gestational days 12 or 18) were isolated from female CD1 mice at 8 weeks of age, followed by isolation of placental and islet total RNA (sections 2.1.2, 2.3, 2.4). mRNAs isolated from mouse placentas on gestational day 12 and 18 as well as mRNAs isolated from islets of non-pregnant, the gestational day 12 and 18 mice were converted into cDNAs (section 2.5). The cDNAs were then analysed utilising RT-PCR to quantify expression of ligand (RSPO1, RSPO2, RSPO3, RSPO4) and receptor (LGR4, LGR5, LGR6) genes (section 2.6). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as a reference gene.

Maternal plasma levels of the RSPO4 protein were measured using a colorimetric mouse RSPO4 ELISA kit (section 2.7).

The effect of the RSPO4 protein on proliferation of MIN6 cells was evaluated using a colorimetric ELISA assay based on BrdU incorporation into DNA (sections 2.8 and 2.9). The effect of the RSPO4 protein on apoptosis of MIN6 cells was analyzed using Caspase Glo® 3/7 assay kit (sections 2.8 and 2.10). The effect of the RSPO4 protein on insulin secretion from MIN6 cells was measured using an insulin secretion assay and a radioimmunoassay (sections 2.11 and 2.12).

5.3 Results

RSPO4 was the only R-spondin gene expressed in the placentas on gestational day 12 whereas on gestational day 18, RSPO1, RSPO3 and RSPO4 expression was observed in the placentas, which was consistent with a role for RSPO4, but not RSPO1 and RSPO3, in adaptive responses of β-cells to pregnancy (Figure 5.1.A). RSPO4 expression in the placentas did not change between gestational day 12 and 18. In addition, RSPO3 was the most highly expressed placental R-spondin gene on gestational day 18, late pregnancy. Since metabolic adaptive responses to pregnancy in the mouse occur during early and mid-pregnancy, RSPO3 is not likely to be involved.

Furthermore, RSPO1, RSPO2, RSPO3 and RSPO4 genes were expressed in non-pregnant pancreatic islets, with RSPO4 as the most highly expressed R-spondin gene (Figure 5.1.C). Expression of RSPO1, RSPO3 and RSPO4, but not RSPO2, was detectable in MIN6 cells, which was consistent with the previously published results [162] (Figure 5.1.B). RSPO4 gene expression in MIN6 cells was higher than expression of RSPO1 and RSPO3.

An analysis of RSPO4 protein concentration in plasma samples of non-pregnant, gestational day 12 and gestational day 18 mice revealed that there was a 6-fold increase in the plasma RSPO4 protein concentration on gestational day 12, compared to the non-pregnant levels (Figure 5.2). There was no significant difference in the RSPO4 protein concentration between non-pregnant and gestational day 18 levels although a rising trend could be observed on day 18. Likewise, there was no significant difference between the gestational day 12 and 18 levels of plasma RSPO4 protein. These data are consistent with the increased placental production and secretion of RSPO4 during pregnancy to influence β-cell function.

The LGR4 gene was the only R-spondin receptor differentially expressed in the islets during pregnancy, compared to non-pregnant gene expression levels (Figure 5.3.A). LGR4 expression dropped both on gestational day 12 and 18 compared to

non-pregnant expression levels but there was no difference between day 12 and 18 expression levels. LGR5 expression was only observed in the islets of non-pregnant mice (Figure 5.3.A). LGR6 expression was not detectable in the islets of both non-pregnant and pregnant mice (Figure 5.3.A). Furthermore, only LGR4, but not LGR5 and LGR6, was expressed in MIN6 cells (Figure 5.3.B). Together, these data suggest that LGR4, but not LGR5 and LGR6, is the islet receptor responsible for transducing the effect of RSPO4 on β-cells during pregnancy.

Analysis of the effect of the RSPO4 protein on BrdU incorporation into DNA of MIN6 cells revealed that RSPO4 caused a concentration-dependent increase in β-cell proliferation, with the maximal effect at 3.5 nM concentration (Figure 5.4). The stimulatory effect of the 3.5 nM RSPO4 protein was more defined in MIN6 cells cultured without FBS-derived growth factors (- FBS) than those cultured in medium supplemented with FBS (+ FBS), which corresponded to an 28% and 16% increase in the β-cell proliferation rate respectively.

In contrast, the RSPO4 protein had no detectable inhibitory effect on β-cell apoptosis. Thus, there was no significant effect of RSPO4 used at 35 pM, 350 pM and 3.5 nM concentrations on MIN6 cell apoptosis (Figure 5.5). While the highest tested RSPO4 protein concentration, 35 nM, resulted in an unexpected upregulation of the MIN6 cell apoptosis rate.

The RSPO4 protein had a small but significant effect on insulin secretion from MIN6 cells. Thus, the 3.5 nM and 35 nM RSPO4 protein significantly increased basal insulin secretion at 2 mM glucose (Figure 5.6). However, the RSPO4 protein did not have any effect on glucose-stimulated insulin secretion from MIN6 cells at 20 mM glucose.

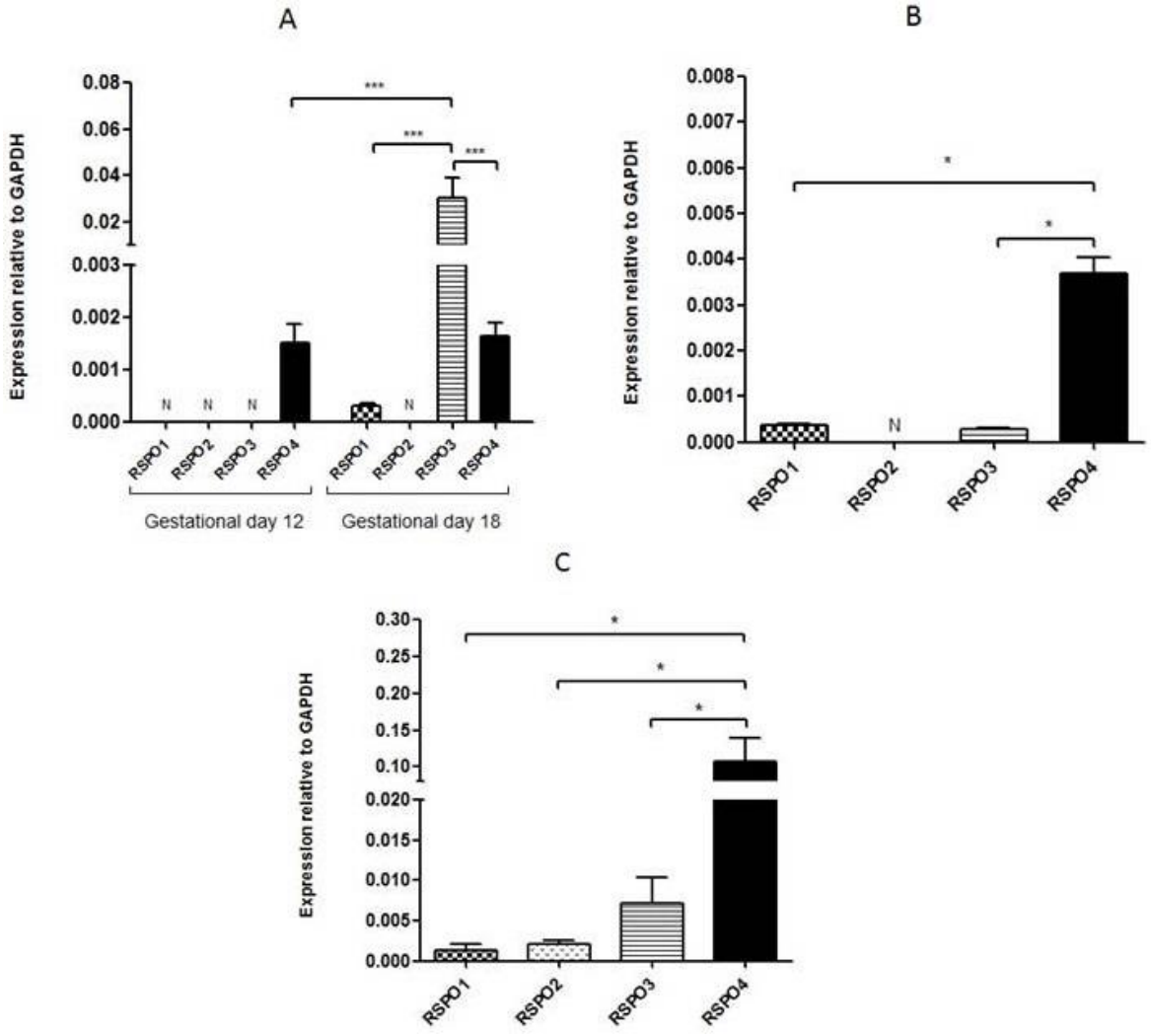

Figure 5.1. Analysis of mouse R-spondin gene family expression on gestational days 12 and 18 in the placentas (A), MIN6 cells (B) and non-pregnant pancreatic islets (C). N – not detectable/not quantifiable expression. Values are expressed as means ± SEM, n = 3-8; two-way ANOVA test (A) or one-way ANOVA test (B and C) * $p<0.05$, *** $p<0.001$.

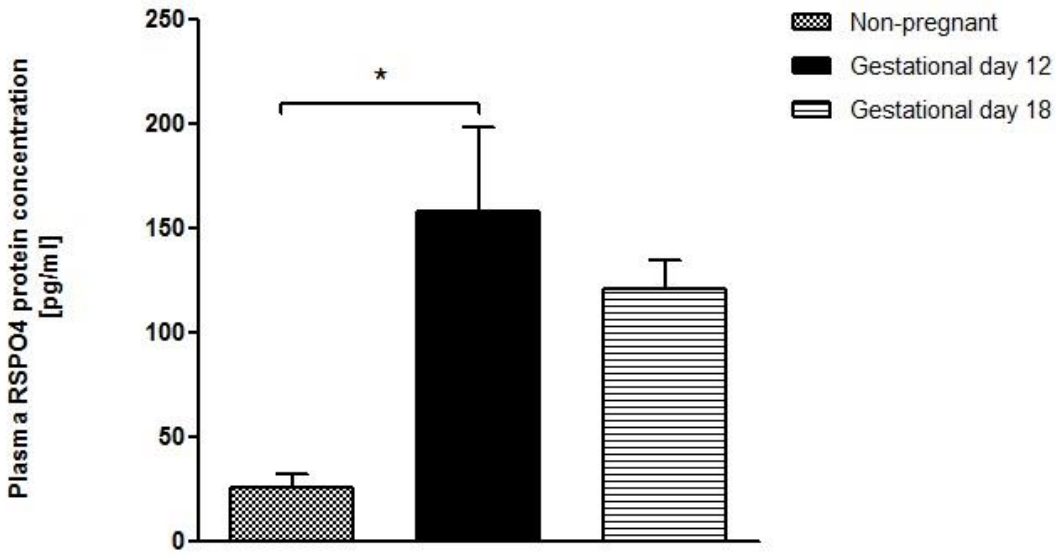

Figure 5.2. Concentration of RSPO4 protein in plasma samples of non-pregnant, gestational day 12 and gestational day 18 mice. Values are expressed as a mean concentration of RSPO4 protein [pg/ml] ± SEM, n = 4-6; one-way ANOVA test * $p<0.05$.

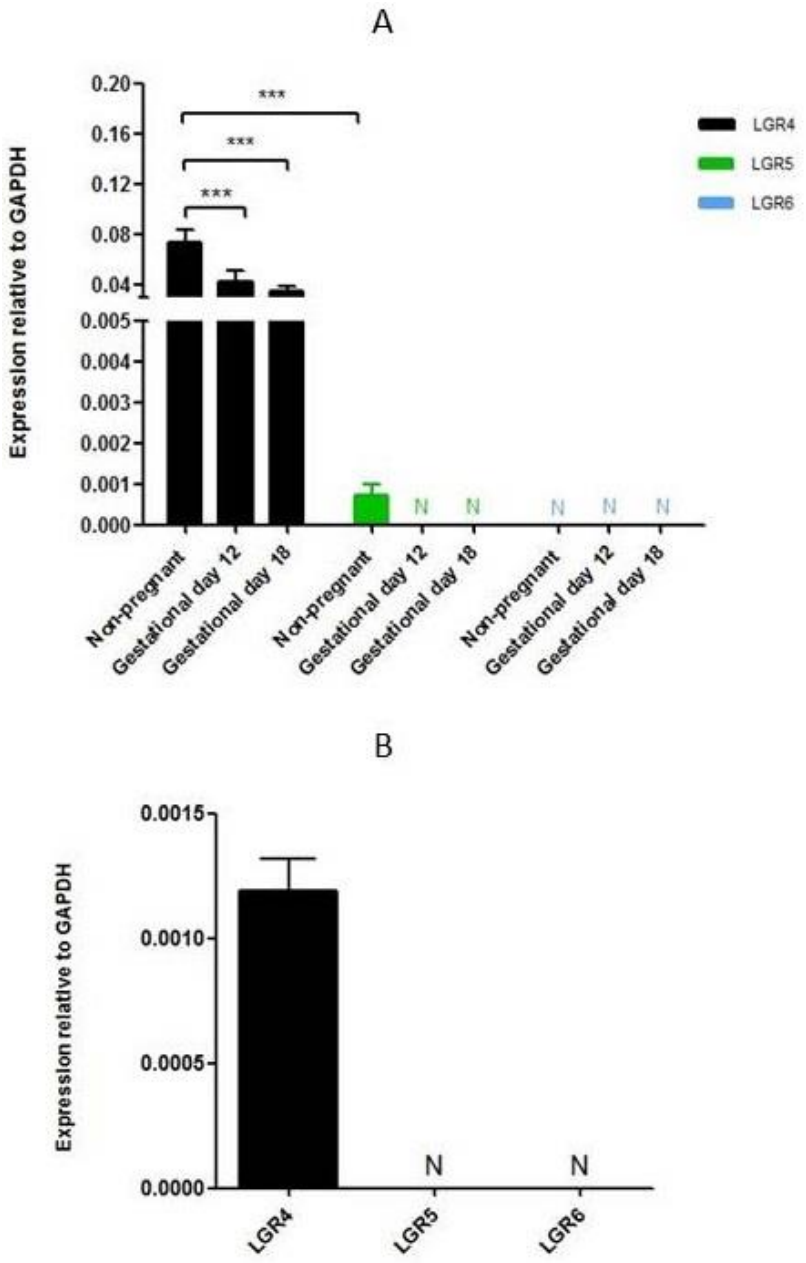

Figure 5.3. Analysis of mouse R-spondin receptors expression in pancreatic islets during pregnancy (A) and in MIN6 cells (B). N – not detectable/not quantifiable expression. Values are expressed as means ± SEM, n = 3-8; two-way ANOVA test *** $p<0.001$.

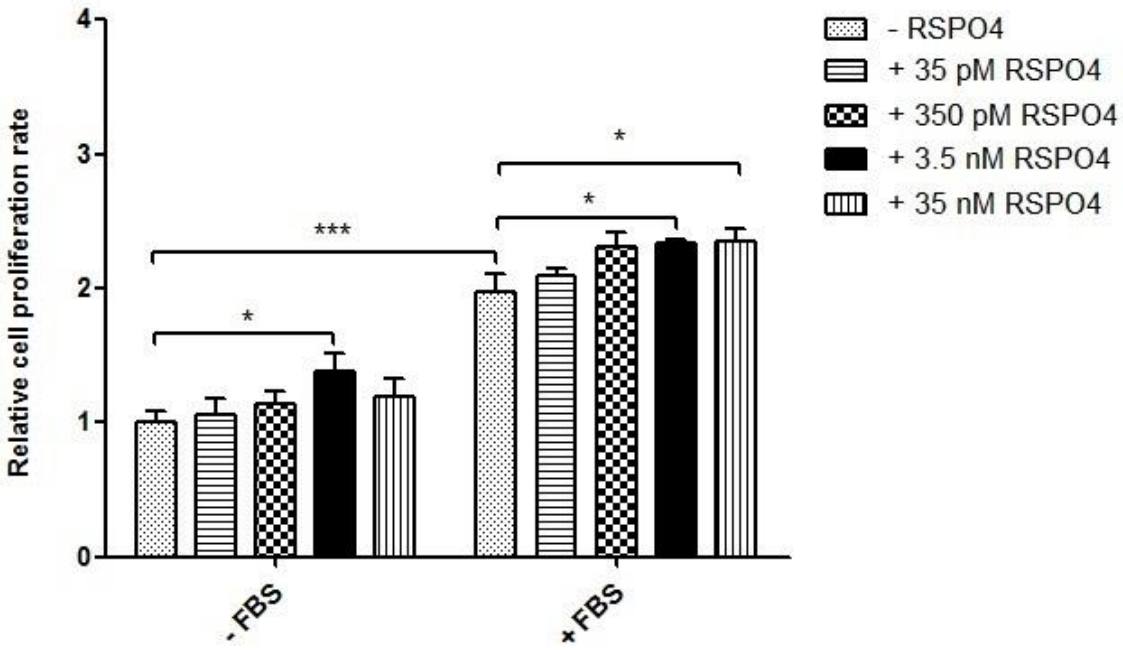

Figure 5.4. The effect of RSPO4 protein on the MIN6 cell proliferation rate. MIN6 cells were cultured in medium containing FBS-derived growth factors (+ FBS) or without them (- FBS); medium without RSPO4 protein (- RSPO4) or medium containing 35 pM RSPO4 protein (+ 35 pM RSPO4), 350 pM RSPO4 protein (+ 350 pM RSPO4), 3.5 nM RSPO4 protein (+ 3.5 nM RSPO4) or 35 nM RSPO4 protein (+ 35 nM RSPO4). Values are expressed as a mean fold change over the control proliferation rate of MIN6 cells cultured in medium without RSPO4 protein and FBS ± SEM, n = 3-8; two-way ANOVA test * $p<0.05$.

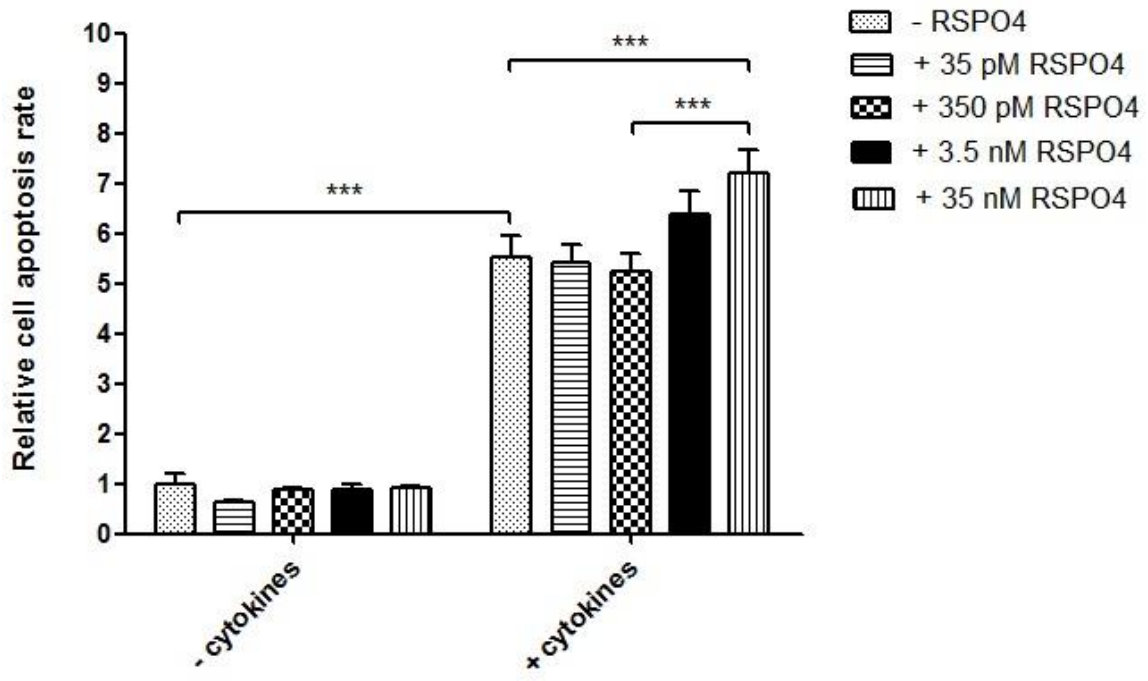

Figure 5.5. The effect of RSPO4 protein on the MIN6 cell apoptosis rate. MIN6 cells were cultured in medium containing pro-apoptotic cytokines (+ cytokines) or without them (- cytokines); medium without RSPO4 protein (- RSPO4) or medium containing 35 pM RSPO4 protein (+ 35 pM RSPO4), 350 pM RSPO4 protein (+ 350 pM RSPO4), 3.5 nM RSPO4 protein (+ 3.5 nM RSPO4) or 35 nM RSPO4 protein (+ 35 nM RSPO4). Values are expressed as a mean fold change over the control apoptosis rate of MIN6 cells cultured in medium without RSPO4 protein and pro-apoptotic cytokines (IL-1β, TNF-α, INF-γ) ± SEM, n = 3-8; two-way ANOVA test * $p<0.05$.

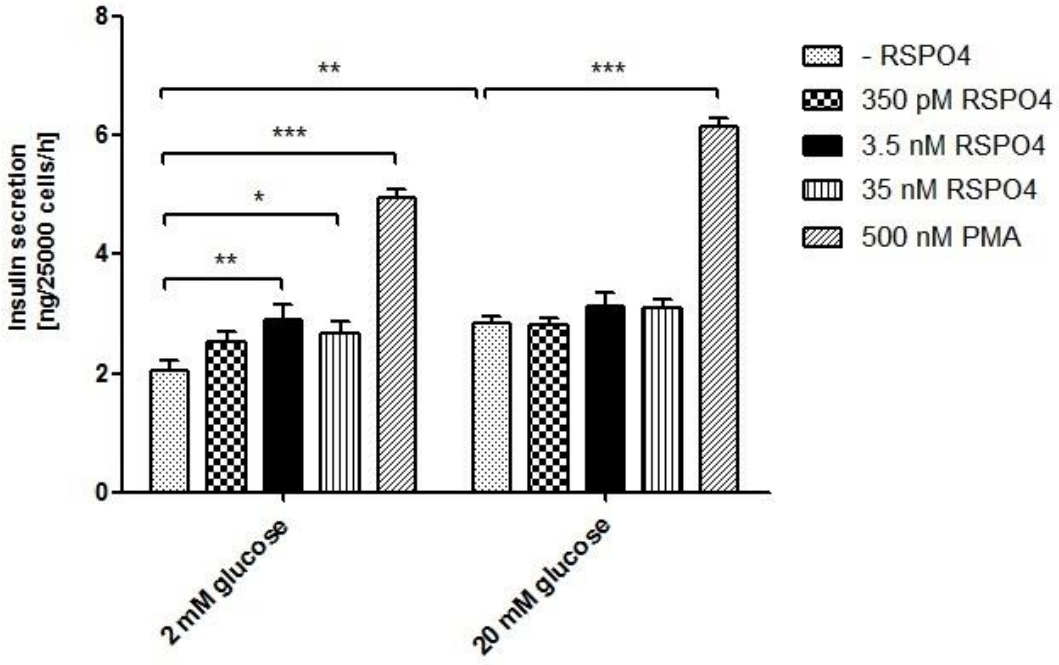

Figure 5.6. The effect of RSPO4 protein on insulin secretion from MIN6 cell. MIN6 cells were cultured in medium containing 2 mM glucose (2 mM glucose) or 20 mM glucose (20 mM glucose); medium without RSPO4 protein (- RSPO4) or medium containing 350 pM RSPO4 protein (+ 350 pM RSPO4), 3.5 nM RSPO4 protein (+ 3.5 nM RSPO4), 35 nM RSPO4 protein (+ 35 nM RSPO4) or 500 nM PMA (500 nM PMA), a PKC activator and stimulator of insulin secretion (positive control). Values are expressed as nanograms of insulin secreted by 25000 MIN6 cells cultured for one hour ± SEM, n = 3-8; two-way ANOVA test * $p<0.05$.

5.4 Discussion

RSPO4 was the only R-spondin gene expressed in the placenta on gestational day 12, when β-cell proliferation is increased in pregnant mice. Furthermore, expression of the LGR4 receptor gene in the islets was downregulated at both the gestational day 12 and 18 compared to non-pregnant expression levels. Therefore, the RSPO4 and its LGR4 receptor expression patterns do not mimic the expression patterns of prolactin and its receptor PRLR, which play a central role in increasing β-cell proliferation during pregnancy. This may suggest that if RSPO4 is involved in the regulation of β-cell function during pregnancy it may have a different role to that of prolactin. RSPO4 has been shown to be a protein secreted by human embryonic kidney 293 cells and mice [146, 156, 169]. In addition, the present study confirmed presence of RSPO4 protein in maternal circulation. Although LGR4 expression was decreased during pregnancy, compared to non-pregnant levels, the RSPO4/LGR4 system may play a positive role in maternal β-cell adaptation as expression levels themselves may not be the best indication of gene involvement in the given physiological process. The GPCR receptor activity can be modulated at many levels, including gene expression, formation of receptor complexes, receptor recycling and degradation. CRLR is a G-protein coupled receptor, which forms heterodimers with the activity-modifying proteins RAMP1, RAMP2 or RAMP3. The CRLR/RAMP1 complex functions as a receptor for CGRP1 whereas CRLR/RAMP2 (AM1) and CRLR/RAMP3 (AM2) complexes mediate adrenomedullin actions [334]. Moreover, CRHR1, a receptor for CRH, interacts with AVP1B, a receptor for AVP, in the brain and pancreatic β-cells, which is manifested by a synergistic upregulation by CRH and AVP of CRH secretion in the brain and insulin in β-cells [242].

The maternal plasma concentration of the RSPO4 protein increased by 6-fold on day 12 compared to the non-pregnant levels. Although, the gestational day 18 RSPO4 protein plasma concentration did not differ significantly from the non-pregnant levels, a rising trend could be observed. Moreover, RSPO4 gene expression was detected in mother's pancreatic islets and MIN6 cells. Those

observations and the fact that RSPO4 has a developmental role suggest that the RSPO4 protein may be secreted by the placenta into the maternal circulation and may exert endocrine and paracrine functions in the mother's body and the fetus [168, 169, 330]. In addition, placental RSPO4 gene expression on day 12 was not significantly different from day 18 expression levels, which mimics the plasma RSPO4 protein concentration similarity at both time points. It is likely that the RSPO4 protein, secreted by the placenta, contributes to the increased RSPO4 protein levels during pregnancy. However, it remains to be determined if the plasma RSPO4 protein is involved in the regulation of β-cell mass expansion during pregnancy.

The lack of RSPO3 expression in the placentas on gestational day 12, with relatively high expression on day 18, suggests that this protein may play a less important role than RSPO4 in the β-cell adaptive responses to pregnancy. Human β-cells, cultured in WNT3A, RSPO3 and Noggin protein conditioned medium, have a 20-fold higher cell proliferation rate than cells cultured in non-conditioned control medium [335]. All four R-spondins stimulate proliferation of small intestine crypt epithelial cells by potentiation of WNT/β-catenin potentiation [146]. Although, RSPO3 expression was not detectable in the placentas on gestational day 12 when β-cell proliferation is increased. Moreover, the WNT3A protein enhances proliferation of MIN6 cells [154]. Noggin stimulates growth and patterning of the neural tube during embryogenesis as well as cartilage morphogenesis and joint formation [336, 337]. Combined actions of RSPO3, WNT3A and Noggin contribute to the enhanced proliferation rate of human β-cells [335].

The RSPO4 protein stimulated proliferation and basal insulin secretion in MIN6 cells. RSPO4 has lower binding affinity to LGR4 and a weaker ability to potentiate WNT/β-catenin signalling compared to the other R-spondins but a significant effect of RSPO4 protein in β-cells was observed in this study [173]. It was previously reported that the 35 nM RSPO1 protein increased MIN6 cell proliferation rate by 2.5-fold while, in this study, concentrations of 3.5 nM and 35 nM RSPO4 protein enhanced the β-cell proliferation rate only by 0.2 to 0.3-fold, depending on the

RSPO4 concentration [162]. However, the concentration of RSPO4 protein measured in the maternal circulation on gestational day 12 was 571 times lower than the lowest most effective RSPO4 concentration which stimulated MIN6 cell proliferation (3.5 nM). The RSPO4 protein also upregulated insulin secretion from MIN6 cells at low glucose (2 mM). On the contrary, the RSPO1 protein has been shown to stimulate insulin secretion from MIN6 cells at both low (2 mM) and high (20 mM) glucose concentrations [162]. The observed differences may be a result of 8-fold higher binding affinity of RSPO1 protein to LGR4 compared to RSPO4 binding [173]. R-spondins potentiate the canonical WNT/β-catenin and non-canonical WNT/PCP signalling pathways, which are implied in embryogenesis, determining cell polarity as well as cell proliferation and survival [157]. Disruption of WNT signalling leads to birth defects and cancer [142]. Experiments on RSPO1 revealed that R-spondins potentiate WNT/β-catenin signalling by a direct interaction of their LGR receptors with the WNT receptor complex FZD and by suppressing FZD complex degradation [159, 181]. Moreover, the WNT3A protein promotes MIN6 cells proliferation while β-cell-specific β-catenin overexpression in mice results in a 2.5-fold increase in β-cell mass [154]. Analysis of R-spondins distribution in the mouse brain revealed that RSPO4 is expressed only in the thalamus while RSPO1 and RSPO3 are expressed more widely in the brain, including the hypothalamus where their expression is downregulated with fasting and upregulated by insulin injection into the third brain ventricle [171]. LGR4 gene expression has also been detected in the hypothalamus. In addition, injection of RSPO1 and RSPO3 into the third brain ventricle, bounded by the hypothalamus and thalamus, results in inhibition of food intake [171]. These observations allow us to assume that R-spondins, in addition to regulating embryogenesis and differentiation, may also be involved in modulating maternal adaptations to pregnancy.

The RSPO4 protein did not have any effect on the basal level of the cell apoptosis rate of MIN6 cells but the highest concentration of the RSPO4 protein (35 nM) unexpectedly potentiated cytokine-induced cell apoptosis, suggesting that lower concentrations of the RSPO4 protein should be used for the future experiments.

Conversely, a protective effect of the 35 nM RSPO1 protein on cytokine-induced MIN6 cell apoptosis has been reported previously [162].

The presence of FBS-derived growth factors diminished the effect of RSPO4 protein on β-cell proliferation. Fetal bovine serum used in cell culture provides a wide range of important biological molecules including albumin, antichymotrpsin, apolipoproteins, biotin, and growth factors, which are essential for optimal cell growth [338]. Transforming growth hormone β1 (TGF-β1), fibroblast growth factor (FGF) and insulin-like growth factor 1 (IGF-1), present in FBS, have a stimulatory effect on the β-cell proliferation rate [339-343]. Cells cultured in "starving" medium, not supplemented with FBS, are more sensitive to potential pro-proliferative or pro-survival factors.

To summarise, RSPO4 fulfils some of the criteria set for determining if a placental ligand may be involved in β-cell adaptive responses to pregnancy. RSPO4 gene was expressed in the placenta during mid-pregnancy (gestational day 12). Its receptor LGR4 was expressed in mouse islets and MIN6 cells, although LGR4 gene expression in the islets during mid- and late pregnancy was lower than in the islets of non-pregnant mice. However, results of experiments presented in Chapter 4 and published by other researchers showed that LGR4 is one of the most highly expressed islet GPCR receptors during mid-pregnancy [331]. The RSPO4 protein concentration is elevated in maternal circulation during mid-pregnancy compared to non-pregnant levels. Furthermore, the RSPO4 protein has pro-proliferative and insulinotropic effects in MIN6 β-cells. Unexpectedly, the RSPO4 does not reduce the rate of MIN6 β-cell apoptosis. Elucidating if RSPO4 plays a part in the regulation of β-cell function during pregnancy requires further research.

Chapter 6

General discussion

Chapter 6

6.1 General discussion

It is estimated that 415 million people worldwide suffered from diabetes mellitus in 2015 and that the number will be 642 million by 2040, making diabetes one of the biggest challenges for medicine in the twenty-first century [344]. Recent studies into the causes of T2DM suggest that the development of the disease depends on inability of the β-cell mass to respond to increased insulin resistance, often as a consequence of obesity [18]. Thus, in normal individuals, increased insulin resistance is compensated for by increased insulin secretion and an increase in the β-cell mass. Individuals who are unable to expand their β-cell mass sufficiently will become glucose intolerant, finally progressing to T2DM. Therapies which are aimed at expanding the β-cell mass therefore offer a targeted way of preventing the onset of T2DM in susceptible, glucose-intolerant patients. However, the mechanisms involved in β-cell adaptive responses to insulin resistance are not well understood, partly because there are few physiological circumstances under which the β-cell mass increases, mainly obesity, which is slow to develop, and pregnancy. During pregnancy, a wide range of adaptive changes is activated in pancreatic β-cells, which is manifested by upregulated cell proliferation and insulin secretion. The modulation of signalling pathways, responsible for the enhanced activity of β-cells during pregnancy, may help to develop new methods for the T2DM treatment. In order to compensate for maternal insulin resistance both humans and rodents activate adaptive mechanisms, involving an increase in β-cell mass and a change in expression of various genes, and the mechanisms of β-cell adaptation to an increased insulin demand in pregnant humans and rodents are thought to be similar. Therefore, research results obtained in rodents could help to broaden our understanding of human β-cell adaptation to pregnancy.

It has been well established that prolactin and placental lactogens play a crucial role in the increase in the β-cell mass during pregnancy, but other physiological signals are likely to play a role as well, and the main aim of the work described in

this thesis was to identify potential candidate molecules. The first aim of the studies was to test the hypothesis that “pregnancy and lactation stimulate β-cell proliferation, with selective loss of new β-cells post-partum” by studying changes in the β-cell mass in pregnant mice. The experiments described in Chapter 3 of this thesis clearly demonstrate that the mouse pregnancy model offers an opportunity to study increases in the β-cell mass under controlled conditions. Therefore, this experimental model was used for subsequent studies investigating potential abilities of placenta-derived signals to regulate β-cell function during pregnancy.

In addition, these studies showed that the post-partum reduction in the β-cell mass is not dependent on the selective loss of “new” β-cells formed during pregnancy. This implies that there is not a sub-set of β-cells that have the capacity to proliferate when physiological circumstances demand, but rather that all islet β-cells have the ability to expand and retract as required by metabolic demand. This information may be important when we consider how best to manipulate the β-cell mass in people with T2DM.

Identifying novel genes involved in the regulation of β-cell function during pregnancy is difficult due to the complexity of direct and indirect effects of GPCR/ligand actions in β-cells. The studies presented in Chapter 4 of this thesis show that there are numerous potential interactions between the placenta and β-cells during pregnancy, determining a number of candidates worth of further investigation for their roles in adaptive responses to pregnancy and for their therapeutic potential in treating T2DM. However, a polygenic disease like T2DM involves small expression changes in a large group of genes, which may be difficult to identify by expression analysis of individual genes. An alternative approach, based on analysis of coordinated changes in gene co-expression networks, can provide molecular insights for complex polygenic diseases [345]. A network-based approach, using global microarray expression data from healthy and T2DM human islets donors, revealed that thrombin receptor F2RL2 gene expression is associated with expression of a module of 192 GPCR genes and

correlates with glycated haemoglobin [318]. On the contrary, F2RL2 expression alone is not altered in patients suffering from T2DM compared to healthy subjects [318].

Using available information about the known functions of the candidate genes is helpful in choosing the most suitable target genes for β-cell functional studies. The most interesting candidate genes would have a pro-proliferative and anti-apoptotic function in various cell types. In addition, knowing which G protein class is coupled to the receptor of interest or which G protein class pathway is activated by a given ligand can help to predict the effect of the analysed gene in β-cells. In general, Gαs and Gαq protein coupling has a stimulatory effect on β-cell proliferation and/or insulin secretion whereas Gαi coupling downregulates those processes in β-cells [102, 105, 113]. Furthermore, the number of known functions of the analysed gene can help to foresee potential side effects of the target gene used as a drug in a therapy for diabetes. Genes involved in regulation of multiple important physiological processes may be more difficult to use as a drug because of a risk of off-target effects and impairment of their innate functions in the body. Therefore, a better option is to choose genes with a narrower range of functions and well-known signalling mechanisms. The fact that the human RSPO4 gene is implicated in inherited anonychia, a mild disorder, and is not implied in other physiological processes, makes it an interesting candidate gene for an anti-diabetes therapy [168, 169, 330].

Stimulation of Gαs protein-dependent and cAMP/PKA signalling has opposing effects in β and α-cells and is useful for developing therapies against T2DM. Pancreatic Gαs protein knockout mice are characterized by decreased proliferation of β-cells and increased proliferation of α-cells, which leads to development of early-onset insulin-deficient diabetes [346, 347]. PKA-mediated phosphorylation of ATP-sensitive potassium channels in INS-1 cells results in plasma membrane depolarization and stimulation of insulin secretion [348]. Moreover, phosphorylation of snapin, a secretory vesicle-associated protein, initiated by GLP-1 analogues and mediated by PKA, promotes insulin exocytosis in mouse islets [349]. However, leptin, through PKA activation, stimulates

trafficking of ATP-dependent potassium channels to the plasma membrane in INS-1 cells, causing inhibition of insulin secretion [350]. Different effects of PKA activation in the same cell type may be related to the subcellular localization of PKA, which depends on binding to A kinase anchoring proteins (AKAPs) [351]. Notably, inactivating mutations in the regulatory subunit of human PKA, responsible for inhibition of the catalytic subunit of the kinase, are linked to enhanced glucose-stimulated insulin secretion, without affecting fasting plasma glucose levels [349]. GLP-1 inhibits glucagon secretion in healthy humans and rats in a PKA-dependent fashion [352-354]. Consequently, GLP-1 administration in patients suffering from T2DM promotes insulin secretion and lowers post-meal plasma glucose levels [355, 356]. Agonists of GLP-1 receptor (GLP1R), a Gαs protein coupled receptor, (exenatide and liraglutide) are used in therapies against T2DM [214, 215]. Interestingly, metformin, used in treatment for T2DM, reduces glucose synthesis in hepatocytes by inhibiting adenylate cyclase, reducing cAMP levels and downregulating PKA activity [357]. Furthermore, blocking of the liver glucagon receptor (GCGR), which is also coupled to Gαs proteins, by its antagonists betters glucose homeostasis in diabetic patients [358].

FFAR1, also known as GPR40, is a Gαq protein-coupled receptor taking part in potentiation of GSIS in the response to stimulation by long-chain free fatty acids (e.g. myristic and palmitic acids) [359]. Moreover, FFAR1 activation in enteroendocrine cells increases GLP-1 release, which indirectly enhances insulin secretion from mouse β-cells [360]. Agonists of FFAR1 augment glucose-dependent insulin secretion in rodents and humans [360-362]. Therefore, targeting FFAR1 by synthetic ligands in diabetic patients has generated promising therapeutic results. Therapies based on some of these agonists have been advanced into phase II or III of clinical trials [362].

Inhibition of Gαi protein-coupled receptors can have therapeutic applications in treating diabetes. Chronic tissue inflammation has been linked to insulin resistance in obese individuals [363]. Inhibiting the pro-inflammatory leukotriene B4 (LTB4) receptor LTB4R1, which is coupled to Gαi proteins, has been effective in reducing

inflammation, improving glucose tolerance and insulin sensitivity in diabetic mice [364]. On the contrary, activation of fractalkine receptor CX3CR1 leads to augmented glucose-, amino acid- and GLP1-induced insulin secretion in mice and isolated human islets, the effects mediated by Gαi protein action [266, 365]. In addition, mutations in the human CX3CR1 gene are associated with an elevated risk of developing T2DM [366]. These findings underline the complex nature of GPCR signalling in β-cells.

Using the selection criteria described in Chapter 4 we decided to investigate RSPO4 as a potential placenta/β-cell signal. The data presented in Chapter 5 suggest that RSPO4 may play a role in regulating β-cell secretory and proliferative responses, at least *in vitro*. These observations for one molecule identified in our screening validate the experimental approach adopted for these studies. Moreover, obtained results demonstrate that placenta-derived molecules other than lactogens and growth hormone are likely to be involved in metabolic responses to pregnancy. Further studies may identify a “cocktail” of biologically active, placenta-derived protein factors which may be used in regulating the β-cell functional mass.

6.2 Future perspectives

The studies described in this thesis have shown that this experimental approach has the potential to identify new biologically active molecules, which may be used to regulate the functional β-cell mass in GDM or, more importantly, in T2DM. RSPO4 function in mouse and human pancreatic islets as well as signalling pathways and receptors involved in RSPO4 action in β-cells require further analysis. As a complementary approach it would be worth assessing the additive or synergistic effects of modulating both stimulatory (Gαs, Gαq) or inhibitory (Gαi) pathways to regulate β-cell functions. Thus, the β-cell effect of downregulating GPCRs coupled to Gαi proteins, which were upregulated in the islets on gestational day 18 compared to the non-pregnant controls, needs further analysis. These GPCRs may be involved in reversing β-cell adaptation to pregnancy at

term, including downregulation of insulin secretion and β-cell mass. Inhibition of Gαi protein-coupled GPCRs may be used to address various aspects of β-cell dysfunction related to T2DM, such as decreased β-cell mass, impaired insulin secretion and signalling [102, 367]. The aforementioned strategies may help to broaden our understanding of β-cell adaptations to pregnancy and an increased insulin demand, equipping us with better tools for designing new anti-diabetes therapies.

Appendix I

List of publications based on this thesis

Research papers

Drynda, R., Peters, C.J., Jones, P.M., Bowe, J.E., *The role of non-placental signals in the adaptation of islets to pregnancy*. Horm Metab Res, 2015. 47(1): p. 64-71.

Posters

Robert Drynda, James Bowe, Peter Jones. *Analysis of mouse placental GPCR ligand secretome during mouse pregnancy*. Poster presentation at Diabetes UK Professional Conference, 2016, Liverpool, UK.

Robert Drynda, Catherine Peters, James Bowe, Peter Jones. *Regulating the loss of β-cell mass post-partum*. Poster presentation at Diabetes UK Professional Conference, 2015, Glasgow, UK.

Robert Drynda, Catherine Peters, James Bowe, Peter Jones. *The role of non-placental signals in the adaptation of islets to pregnancy.* Poster presentation at Diabetes UK Professional Conference, 2014, Liverpool, UK.

Appendix II

Table A2.1. List of mouse placental GPCR ligand gene primers.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
ADCYAP1	Adenylate Cyclase Activating Polypeptide 1	QT00100317	92
ADM	Adrenomedullin	QT00249676	105
ADM2	Adrenomedullin 2	QT00269003	150
AGT	Angiotensinogen	QT00164745	61
ANXA1	Annexin A1	QT00145915	170
APLN	Apelin	QT00111762	103
AVP	Arginine Vasopressin	QT00249389	93
BGLAP	Bone Gamma-Carboxyglutamate (Gla) Protein	QT00259406	102
C1QL1	Complement Component 1, Q Subcomponent-Like 1	QT00259238	90
C1QL2	Complement Component 1, Q Subcomponent-Like 2	QT00318227	126
C1QL3	Complement Component 1, Q Subcomponent-Like 3	QT00282478	64
C1QL4	Complement Component 1, Q Subcomponent-Like 4	QT01038520	100
C3	Complement Component 3	QT00109270	108
C4A	Complement Component 4A	QT01074948	187
CALCA	Calcitonin-Related Polypeptide Alpha	QT01747865	96
CALCB	Calcitonin-Related Polypeptide Beta	QT00162078	94
CARTPT	CART Prepropeptide	QT00130396	82
CCK	Cholecystokinin	QT00250145	91
CCL1	Chemokine (C-C Motif) Ligand 1	QT01044421	111
CCL11	Chemokine (C-C Motif) Ligand 11	QT00114275	109
CCL17	Chemokine (C-C Motif) Ligand 17	QT00131572	103
CCL19	Chemokine (C-C Motif) Ligand 19	QT02532173	68
CCL2	Chemokine (C-C Motif) Ligand 2	QT00167832	118
CCL20	Chemokine (C-C Motif) Ligand 20	QT02326394	71
CCL21a	Chemokine (C-C Motif) Ligand 21A	QT00284753	96
CCL22	Chemokine (C-C Motif) Ligand 22	QT00108031	92
CCL24	Chemokine (C-C Motif) Ligand 24	QT00126021	116
CCL25	Chemokine (C-C Motif) Ligand 25	QT00110173	143
CCL26	Chemokine (C-C Motif) Ligand 26	QT01559481	99
CCL27a	Chemokine (C-C Motif) Ligand 27A	QT00325003	190
CCL27b	Chemokine (C-C Motif) Ligand 27B	QT02281895	113
CCL28	Chemokine (C-C Motif) Ligand 28	QT01752625	102
CCL3	Chemokine (C-C Motif) Ligand 3	QT00248199	107
CCL4	Chemokine (C-C Motif) Ligand 4	QT00154616	110

Table A2.1. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
CCL5	Chemokine (C-C Motif) Ligand 5	QT01747165	107
CCL7	Chemokine (C-C Motif) Ligand 7	QT00171458	117
CCL8	Chemokine (C-C Motif) Ligand 8	QT00128548	87
CD55	CD55 Molecule	QT00133994	121
CGA	Glycoprotein Hormones, Alpha Polypeptide	QT00098084	81
COL3A1	Collagen Type III, Alpha 1	QT02331301	73
CORT	Cortistatin	QT00104272	149
CRH	Corticotropin Releasing Hormone	QT01055789	183
CTSG	Cathepsin G	QT00112112	118
CX3CL1	Chemokine (C-X3-C Motif) Ligand 1	QT00128345	98
CXCL1	Chemokine (C-X-C Motif) Ligand 1	QT00115647	93
CXCL10	Chemokine (C-X-C Motif) Ligand 10	QT00093436	92
CXCL11	Chemokine (C-X-C Motif) Ligand 11	QT00265041	86
CXCL12	Chemokine (C-X-C Motif) Ligand 12	QT00161112	71
CXCL13	Chemokine (C-X-C Motif) Ligand 13	QT00107919	64
CXCL16	Chemokine (C-X-C Motif) Ligand 16	QT00171535	129
CXCL2	Chemokine (C-X-C Motif) Ligand 2	QT00113253	81
CXCL3	Chemokine (C-X-C Motif) Ligand 3	QT00151599	133
CXCL4	Chemokine (C-X-C Motif) Ligand 4	QT00312529	82
CXCL5	Chemokine (C-X-C Motif) Ligand 5	QT01658146	62
CXCL9	Chemokine (C-X-C Motif) Ligand 9	QT00097062	98
EDN1	Endothelin 1	QT00253512	116
EDN2	Endothelin 2	QT00198814	77
EDN3	Endothelin 3	QT00107905	140
F2	Thrombin	QT00128870	137
FSHB	Follicle Stimulating Hormone, Beta Polypeptide	QT00150262	109
GAL	Galanin	QT00109970	69
GALP	Galanin-Like Peptide	QT02532796	121
GAST	Gastrin	QT00107702	150
GCG	Glucagon	QT00124033	94
GHRH	Growth Hormone Releasing Hormone	QT00136787	121
GHRL	Ghrelin/Obestatin Prepropeptide	QT00137536	73
GIP	Gastric Inhibitory Polypeptide	QT00146692	140
GNRH1	Gonadotropin-Releasing Hormone 1	QT01062600	124
GRP	Gastrin-Releasing Peptide	QT00101780	79

Table A2.1. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
HCRT	Hypocretin (Orexin) Neuropeptide Precursor	QT00112252	156
INSL3	Insulin-Like 3	QT00286741	148
INSL5	Insulin-Like 5	QT00261345	146
KISS1	Kisspeptin	QT01764259	146
KNG1	Kininogen 1	QT00106260	99
LHB	Luteinizing Hormone Beta Polypeptide	QT00262829	150
NMB	Neuromedin B	QT00105945	92
NMS	Neuromedin S	QT01043672	127
NMU	Neuromedin U	QT00133091	100
NPFF	Neuropeptide FF-Amide Peptide Precursor	QT00319984	120
NPS	Neuropeptide S	QT02251396	69
NPVF	Neuropeptide VF Precursor	QT01054340	118
NPW	Neuropeptide W	QT00295400	119
NPY	Neuropeptide Y	QT00134302	150
NTS	Neurotensin	QT00127225	142
OXT	Oxytocin/Neurophysin I Prepropeptide	QT00252203	184
PDYN	Prodynorphin	QT00110117	143
PENK	Proenkephalin	QT00240961	95
PMCH	Pro-Melanin-Concentrating Hormone	QT01060339	106
PNOC	Prepronociceptin	QT00102480	85
POMC	Proopiomelanocortin	QT00162218	99
PPBP	Pro-Platelet Basic Protein	QT00160993	96
PPY	Pancreatic Polypeptide	QT00103999	150
PRLH	Prolactin Releasing Hormone	QT01564941	99
PROK1	Prokineticin 1	QT02326296	184
PROK2	Prokineticin 2	QT00133910	62
PRSS1	Trypsin I	QT01757651	113
PTH	Parathyroid Hormone	QT00161539	96
PTH2	Parathyroid Hormone 2	QT00284102	80
PTHLH	Parathyroid Hormone-Like Hormone	QT00114982	119
PYY	Peptide YY	QT00123277	79
QRFP	Pyroglutamylated RFamide Peptide	QT00136395	83
RARRES2	Chemerin	QT00117166	84
RLN1	Relaxin 1	QT01071112	73
RLN3	Relaxin 3	QT01073996	97

Table A2.1. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
RSPO1	R-spondin 1	QT00124831	67
RSPO2	R-spondin 2	QT00154182	129
RSPO3	R-spondin 3	QT00127988	138
RSPO4	R-spondin 4	QT00136234	119
SCT	Secretin	QT00249361	134
SST	Somatostatin	QT01046528	89
TAC1	Preprotachykinin	QT01037141	154
TRH	Thyrotropin-Releasing Hormone	QT01745023	109
TSHB	Thyroid Stimulating Hormone Beta	QT00135303	95
UCN	Urocortin	QT00326879	112
UCN2	Urocortin 2	QT01556534	125
UCN3	Urocortin 3	QT00302267	125
UTS2	Urotensin 2	QT00124278	92
VIP	Vasoactive Intestinal Peptide	QT00133966	96
WNT1	Wingless-Type MMTV Integration Site Family Member 1	QT00103985	86
WNT2	Wingless-Type MMTV Integration Site Family Member 2	QT00118503	119
WNT3	Wingless-Type MMTV Integration Site Family Member 3	QT00133686	71
WNT3A	Wingless-Type MMTV Integration Site Family Member 3A	QT00250439	94
WNT4	Wingless-Type MMTV Integration Site Family Member 4	QT00104622	134
WNT5A	Wingless-Type MMTV Integration Site Family Member 5A	QT00164500	130
WNT7B	Wingless-Type MMTV Integration Site Family Member 7B	QT00168812	114
XCL1	Chemokine (C Motif) Ligand 1	QT00095228	69

Table A2.2. List of mouse pancreatic islet GPCR gene primers.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
ADCYAP1R1	Adenylate Cyclase Activating Polypeptide 1 Receptor Type I	QT00120561	115
ADORA1	Adenosine A1 Receptor	QT00301119	68
ADORA2A	Adenosine A2a Receptor	QT02527308	112
ADORA2B	Adenosine A2b Receptor	QT00257558	112
ADORA3	Adenosine A3 Receptor	QT01068347	142
ADRA1A	Adrenoceptor Alpha 1A	QT00129584	100
ADRA1B	Adrenoceptor Alpha 1B	QT01051876	89
ADRA1D	Adrenoceptor Alpha 1D	QT00147315	99
ADRA2A	Adrenoceptor Alpha 2A	QT00287063	60
ADRA2B	Adrenoceptor Alpha 2B	QT00311675	83
ADRA2C	Adrenoceptor Alpha 2C	QT01749328	95
ADRB1	Adrenoceptor Beta 1	QT00258692	109
ADRB2	Adrenoceptor Beta 2	QT00253967	85
ADRB3	Adrenoceptor Beta 3	QT01756160	102
AGTR1	Angiotensin II Receptor Type 1	QT00261464	85
AGTR2	Angiotensin II Receptor Type 2	QT00197540	81
APLNR	Apelin Receptor	QT00254275	92
AVPR1A	Arginine Vasopressin Receptor 1A	QT00113169	133
AVPR1B	Arginine Vasopressin Receptor 1B	QT01753941	134
AVPR2	Arginine Vasopressin Receptor 2	QT00315315	102
BAI1	Adhesion G Protein-Coupled Receptor B1	QT00123228	85
BAI2	Adhesion G Protein-Coupled Receptor B2	QT00158732	129
BAI3	Adhesion G Protein-Coupled Receptor B3	QT01062873	117
BDKRB1	Bradykinin Receptor B1	QT00326886	70
BDKRB2	Bradykinin Receptor B2	QT00111027	84
BRS3	Bombesin-Like Receptor 3	QT00250222	100
C3AR1	Complement Component 3a Receptor 1	QT02380581	150
C5AR1	Complement Component 5a Receptor 1	QT01164723	119
CALCR	Calcitonin Receptor	QT00108864	100
CALCRL	Calcitonin Receptor-Like	QT00128786	146
CASR	Calcium-Sensing Receptor	QT00134015	61
CCBP2	Atypical Chemokine Receptor 2	QT00154105	129
CCKAR	Cholecystokinin A Receptor	QT00099673	101
CCKBR	Cholecystokinin B Receptor	QT00128597	122
CCR1	Chemokine (C-C Motif) Receptor 1	QT00156058	139

Table A2.2. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
CCR10	Chemokine (C-C Motif) Receptor 10	QT00260673	125
CCR2	Chemokine (C-C Motif) Receptor 2	QT02522849	149
CCR3	Chemokine (C-C Motif) Receptor 3	QT00262822	109
CCR4	Chemokine (C-C Motif) Receptor 4	QT00250432	146
CCR5	Chemokine (C-C Motif) Receptor 5	QT00114569	60
CCR6	Chemokine (C-C Motif) Receptor 6	QT02379181	120
CCR7	Chemokine (C-C Motif) Receptor 7	QT00240975	95
CCR8	Chemokine (C-C Motif) Receptor 8	QT01059184	82
CCR9	Chemokine (C-C Motif) Receptor 9	QT01165038	67
CCRL2	Chemokine (C-C Motif) Receptor-Like 2	QT01040179	114
CD97	Adhesion G Protein-Coupled Receptor E5	QT00198919	104
CELSR1	Cadherin EGF LAG Seven-Pass G-Type Receptor 1	QT01748096	89
CELSR2	Cadherin EGF LAG Seven-Pass G-Type Receptor 2	QT01536724	100
CELSR3	Cadherin EGF LAG Seven-Pass G-Type Receptor 3	QT00123865	108
CHRM1	Cholinergic Receptor Muscarinic 1	QT01753311	112
CHRM2	Cholinergic Receptor Muscarinic 2	QT00290297	93
CHRM3	Cholinergic Receptor Muscarinic 3	QT01772169	141
CHRM4	Cholinergic Receptor Muscarinic 4	QT00313712	86
CHRM5	Cholinergic Receptor Muscarinic 5	QT00298452	100
CMKLR1	Chemerin Chemokine-Like Receptor 1	QT01060430	91
CNR1	Cannabinoid Receptor 1	QT02522457	163
CNR2	Cannabinoid Receptor 2	QT00159558	150
CRHR1	Corticotropin Releasing Hormone Receptor 1	QT00106232	117
CRHR2	Corticotropin Releasing Hormone Receptor 2	QT00151543	75
CX3CR1	Chemokine (C-X3-C Motif) Receptor 1	QT00259126	63
CXCR1	Chemokine (C-X-C Motif) Receptor 1	QT01778434	100
CXCR2	Chemokine (C-X-C Motif) Receptor 2	QT00283696	80
CXCR3	Chemokine (C-X-C Motif) Receptor 3	QT00249438	109
CXCR4	Chemokine (C-X-C Motif) Receptor 4	QT00249305	106
CXCR5	Chemokine (C-X-C Motif) Receptor 5	QT00253449	118
CXCR6	Chemokine (C-X-C Motif) Receptor 6	QT02528806	135
CXCR7	Chemokine (C-X-C Motif) Receptor 7	QT00254443	88
CYSLTR1	Cysteinyl Leukotriene Receptor 1	QT01756503	68
CYSLTR2	Cysteinyl Leukotriene Receptor 2	QT00136885	99
DARC	Atypical Chemokine Receptor 1	QT01748663	62

Table A2.2. Continued.

Gene symbol	Gene name	Primer Asssay Qiagen Catalogue Number	Amplicon length (bp)
DRD1A	Dopamine Receptor D1	QT00263396	104
DRD2	Dopamine Receptor D2	QT01169063	126
DRD3	Dopamine Receptor D3	QT00170527	78
DRD4	Dopamine Receptor D4	QT00125489	137
DRD5	Dopamine Receptor D5	QT00281596	142
EDNRA	Endothelin Receptor Type A	QT00121625	101
EDNRB	Endothelin Receptor Type B	QT00139384	129
ELTD1	EGF Latrophilin And Seven Transmembrane Domain-Containing Protein 1	QT00170051	92
EMR1	Egf-Like Module Containing Mucin-Like Hormone Receptor-Like 1	QT00099617	87
F2R	Coagulation Factor II (Thrombin) Receptor	QT00119812	125
F2RL1	Coagulation Factor II (Thrombin) Receptor-Like 1	QT02255330	88
F2RL2	Coagulation Factor II (Thrombin) Receptor-Like 2	QT00101178	146
F2RL3	Coagulation Factor II (Thrombin) Receptor-Like 3	QT00252168	129
FFAR1	Free Fatty Acid Receptor 1	QT00308833	70
FFAR2	Free Fatty Acid Receptor 2	QT00128226	61
FFAR3	Free Fatty Acid Receptor 3	QT00303499	99
FPR1	Formyl Peptide Receptor 1	QT01165899	123
FPR2	Formyl Peptide Receptor 2	QT00171514	73
FPR3	Formyl Peptide Receptor 3	QT01063216	111
FSHR	Follicle Stimulating Hormone Receptor	QT00122472	92
FZD1	Frizzled Class Receptor 1	QT00290542	126
FZD10	Frizzled Class Receptor 10	QT00279979	72
FZD2	Frizzled Class Receptor 2	QT00261485	148
FZD3	Frizzled Class Receptor 3	QT00147917	101
FZD4	Frizzled Class Receptor 4	QT00260526	125
FZD5	Frizzled Class Receptor 5	QT01063461	149
FZD6	Frizzled Class Receptor 6	QT00109998	98
FZD7	Frizzled Class Receptor 7	QT00307797	120
FZD8	Frizzled Class Receptor 8	QT02328151	83
FZD9	Frizzled Class Receptor 9	QT00248906	108
GABBR1	Gamma-Aminobutyric Acid (GABA) B Receptor 1	QT01052541	114
GABBR2	Gamma-Aminobutyric Acid (GABA) B Receptor 2	QT01045912	105
GALR1	Galanin Receptor 1	QT00108969	107
GALR2	Galanin Receptor 2	QT00262836	61
GALR3	Galanin Receptor 3	QT00326081	113

Table A2.2. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
GCGR	Glucagon Receptor	QT00112560	84
GHRHR	Growth Hormone Releasing Hormone Receptor	QT01062243	107
GHSR	Growth Hormone Secretagogue Receptor	QT00138439	121
GIPR	Gastric Inhibitory Polypeptide Receptor	QT01564913	132
GLP2R	Glucagon-Like Peptide 2 Receptor	QT01744197	116
GNRHR	Gonadotropin-Releasing Hormone Receptor	QT00107205	109
GPBAR1	G Protein-Coupled Bile Acid Receptor 1	QT01038471	95
GPER	G Protein-Coupled Estrogen Receptor 1	QT00260659	110
GPR1	G Protein-Coupled Receptor 1	QT00124341	110
GPR101	G Protein-Coupled Receptor 101	QT00309512	89
GPR110	G Protein-Coupled Receptor 110	QT00198170	62
GPR111	G Protein-Coupled Receptor 111	QT01162679	121
GPR112	G Protein-Coupled Receptor 112	QT02327584	119
GPR113	G Protein-Coupled Receptor 113	QT01168748	140
GPR114	G Protein-Coupled Receptor 114	QT01161699	75
GPR115	G Protein-Coupled Receptor 115	QT01753808	91
GPR116	G Protein-Coupled Receptor 116	QT01070272	77
GPR119	G Protein-Coupled Receptor 119	QT01758953	99
GPR12	G Protein-Coupled Receptor 12	QT01062656	140
GPR120	G Protein-Coupled Receptor 120	QT00257033	97
GPR123	G Protein-Coupled Receptor 123	QT01069404	94
GPR124	G Protein-Coupled Receptor 124	QT00129731	91
GPR125	G Protein-Coupled Receptor 125	QT01057294	88
GPR126	G Protein-Coupled Receptor 126	QT01065862	145
GPR128	G Protein-Coupled Receptor 128	QT02248449	110
GPR132	G Protein-Coupled Receptor 132	QT00249900	103
GPR133	G Protein-Coupled Receptor 133	QT01775907	105
GPR135	G Protein-Coupled Receptor 135	QT01040977	137
GPR137	G Protein-Coupled Receptor 137	QT00158263	73
GPR139	G Protein-Coupled Receptor 139	QT00306803	111
GPR141	G Protein-Coupled Receptor 141	QT01163561	85
GPR142	G Protein-Coupled Receptor 142	QT01040298	66
GPR143	G Protein-Coupled Receptor 143	QT00494613	126
GPR144	G Protein-Coupled Receptor 144	QT02303084	90
GPR146	G Protein-Coupled Receptor 146	QT00292726	131

Table A2.2. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
GPR149	G Protein-Coupled Receptor 149	QT00123578	116
GPR15	G Protein-Coupled Receptor 15	QT00283717	77
GPR150	G Protein-Coupled Receptor 150	QT00279293	98
GPR151	G Protein-Coupled Receptor 151	QT00281085	136
GPR152	G Protein-Coupled Receptor 152	QT00267477	83
GPR153	G Protein-Coupled Receptor 153	QT00117859	98
GPR156	G Protein-Coupled Receptor 156	QT00140784	73
GPR157	G Protein-Coupled Receptor 157	QT01061284	135
GPR158	G Protein-Coupled Receptor 158	QT01059142	101
GPR160	G Protein-Coupled Receptor 160	QT01075375	90
GPR161	G Protein-Coupled Receptor 161	QT01064399	81
GPR162	G Protein-Coupled Receptor 162	QT00098973	68
GPR17	G Protein-Coupled Receptor 17	QT01043707	77
GPR171	G Protein-Coupled Receptor 171	QT01050700	96
GPR173	G Protein-Coupled Receptor 173	QT01164716	111
GPR174	G Protein-Coupled Receptor 174	QT01542695	149
GPR176	G Protein-Coupled Receptor 176	QT00126532	78
GPR179	G Protein-Coupled Receptor 179	QT02243598	84
GPR18	G Protein-Coupled Receptor 18	QT00129227	129
GPR182	G Protein-Coupled Receptor 182	QT01163666	87
GPR183	G Protein-Coupled Receptor 183	QT00295785	100
GPR19	G Protein-Coupled Receptor 19	QT02242415	106
GPR20	G Protein-Coupled Receptor 20	QT00266735	141
GPR21	G Protein-Coupled Receptor 21	QT00322035	108
GPR22	G Protein-Coupled Receptor 22	QT00131775	63
GPR25	G Protein-Coupled Receptor 25	QT02270226	109
GPR26	G Protein-Coupled Receptor 26	QT00285215	68
GPR27	G Protein-Coupled Receptor 27	QT01062754	105
GPR3	G Protein-Coupled Receptor 3	QT00249732	92
GPR34	G Protein-Coupled Receptor 34	QT00168938	101
GPR35	G Protein-Coupled Receptor 35	QT00495411	107
GPR37	G Protein-Coupled Receptor 37	QT01062747	95
GPR37L1	G Protein-Coupled Receptor 37 Like 1	QT00157409	77
GPR39	G Protein-Coupled Receptor 39	QT00315028	140
GPR4	G Protein-Coupled Receptor 4	QT00264243	103

Table A2.2. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
GPR45	G Protein-Coupled Receptor 45	QT00258657	99
GPR50	G Protein-Coupled Receptor 50	QT00124117	98
GPR52	G Protein-Coupled Receptor 52	QT01071847	148
GPR56	G Protein-Coupled Receptor 56	QT01060472	98
GPR6	G Protein-Coupled Receptor 6	QT00296044	119
GPR61	G Protein-Coupled Receptor 61	QT00125755	97
GPR62	G Protein-Coupled Receptor 62	QT00302393	67
GPR63	G Protein-Coupled Receptor 63	QT00136920	73
GPR64	G Protein-Coupled Receptor 64	QT00142149	131
GPR65	G Protein-Coupled Receptor 65	QT00144774	84
GPR68	G Protein-Coupled Receptor 68	QT02392397	85
GPR75	G Protein-Coupled Receptor 75	QT02530157	154
GPR77	G Protein-Coupled Receptor 77	QT02532803	87
GPR82	G Protein-Coupled Receptor 82	QT00126784	144
GPR83	G Protein-Coupled Receptor 83	QT00104216	85
GPR84	G Protein-Coupled Receptor 84	QT00279888	117
GPR85	G Protein-Coupled Receptor 85	QT00281022	128
GPR87	G Protein-Coupled Receptor 87	QT01038856	150
GPR88	G Protein-Coupled Receptor 88	QT02263681	121
GPR97	G Protein-Coupled Receptor 97	QT01053227	169
GPR98	G Protein-Coupled Receptor 98	QT01061340	95
GPRC5A	G Protein-Coupled Receptor Class C Group 5 Member A	QT00104832	141
GPRC5B	G Protein-Coupled Receptor Class C Group 5 Member B	QT00124348	75
GPRC5C	G Protein-Coupled Receptor Class C Group 5 Member C	QT00148582	157
GPRC5D	G Protein-Coupled Receptor Class C Group 5 Member D	QT00132363	93
GPRC6A	G Protein-Coupled Receptor Class C Group 5 Member 6A	QT00147098	149
GRM1	Glutamate Receptor Metabotropic 1	QT00175042	84
GRM2	Glutamate Receptor Metabotropic 2	QT02327822	123
GRM3	Glutamate Receptor Metabotropic 3	QT00171542	68
GRM4	Glutamate Receptor Metabotropic 4	QT01072855	60
GRM5	Glutamate Receptor Metabotropic 5	QT01552117	146
GRM6	Glutamate Receptor Metabotropic 6	QT00133525	148
GRM7	Glutamate Receptor Metabotropic 7	QT01167509	83
GRM8	Glutamate Receptor Metabotropic 8	QT00169267	92
GRPR	Gastrin-Releasing Peptide Receptor	QT00248885	88

Table A2.2. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
HCRTR1	Hypocretin (Orexin) Receptor 1	QT01755054	128
HCRTR2	Hypocretin (Orexin) Receptor 2	QT00171430	89
HRH1	Histamine Receptor H1	QT01161300	109
HRH2	Histamine Receptor H2	QT01039157	80
HRH3	Histamine Receptor H3	QT00158375	85
HRH4	Histamine Receptor H4	QT00135884	150
HTR1A	5-hydroxytryptamine receptor 1A	QT00250516	76
HTR1B	5-hydroxytryptamine receptor 1B	QT01039913	119
HTR1D	5-hydroxytryptamine receptor 1D	QT01759156	101
HTR1F	5-hydroxytryptamine receptor 1F	QT00102242	76
HTR2A	5-hydroxytryptamine receptor 2A	QT00282947	125
HTR2B	5-hydroxytryptamine receptor 2B	QT00144704	151
HTR2C	5-hydroxytryptamine receptor 2C	QT00144403	175
HTR4	5-hydroxytryptamine receptor 4	QT00108143	104
HTR5A	5-hydroxytryptamine receptor 5A	QT00248290	133
HTR6	5-hydroxytryptamine receptor 6	QT00261870	126
HTR7	5-hydroxytryptamine receptor 7	QT00150346	100
KISS1R	Kisspeptins Receptor	QT00140427	123
LGR4	Leucine-Rich Repeat Containing G Protein-Coupled Receptor 4	QT01065295	99
LGR5	Leucine-Rich Repeat Containing G Protein-Coupled Receptor 5	QT00123193	109
LGR6	Leucine-Rich Repeat Containing G Protein-Coupled Receptor 6	QT02525292	93
LHCGR	Luteinizing Hormone/Choriogonadotropin Receptor	QT00101990	83
LPAR1	Lysophosphatidic Acid Receptor 1	QT00107709	94
LPAR2	Lysophosphatidic Acid Receptor 2	QT00106008	94
LPAR3	Lysophosphatidic Acid Receptor 3	QT00264320	99
LPAR4	Lysophosphatidic Acid Receptor 4	QT00125888	96
LPAR5	Lysophosphatidic Acid Receptor 5	QT00312571	100
LPAR6	Lysophosphatidic Acid Receptor 6	QT00325668	118
LPHN1	Calcium-Independent Alpha-Latrotoxin Receptor 1	QT01048292	91
LPHN2	Calcium-Independent Alpha-Latrotoxin Receptor 2	QT02284037	119
LPHN3	Calcium-Independent Alpha-Latrotoxin Receptor 3	QT01066856	104
LTB4R	Leukotriene B4 Receptor	QT00197897	110
LTB4R2	Leukotriene B4 Receptor 2	QT01054935	158
MAS1	Mas-Related G Protein-Coupled Receptor A	QT02257157	72
MC1R	Melanocortin 1 Receptor	QT00305011	110

Table A2.2. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
MC2R	Melanocortin 2 Receptor	QT01066338	89
MC3R	Melanocortin 3 Receptor	QT00264404	108
MC4R	Melanocortin 4 Receptor	QT00280861	106
MC5R	Melanocortin 5 Receptor	QT01166494	90
MCHR1	Melanin-Concentrating Hormone Receptor 1	QT00312291	134
MRGPRD	MAS-Related GPR Member D	QT01078917	115
MRGPRE	MAS-Related GPR Member E	QT00282205	80
MRGPRF	MAS-Related GPR Member F	QT01078924	126
MRGPRG	MAS-Related GPR Member G	QT00298480	77
MRGPRX1	MAS-Related GPR Member X1	QT02330237	67
MRGPRX2	MAS-Related GPR Member X2	QT02530808	139
MTNR1A	Melatonin Receptor 1A	QT00253029	82
MTNR1B	Melatonin Receptor 1B	QT01040648	84
NMBR	Neuromedin B Receptor	QT00312494	112
NMUR1	Neuromedin U Receptor 1	QT00174006	110
NMUR2	Neuromedin U Receptor 2	QT00137998	106
NPBWR1	Neuropeptides B/W Receptor 1	QT00299040	84
NPFFR1	Neuropeptide FF Receptor 1	QT02528113	113
NPFFR2	Neuropeptide FF Receptor 2	QT00303975	69
NPSR	Neuropeptide S Receptor 1	QT00152180	121
NPY1R	Neuropeptide Y Receptor Y1	QT00249746	134
NPY2R	Neuropeptide Y Receptor Y2	QT01744036	91
NPY5R	Neuropeptide Y Receptor Y5	QT00109088	146
NTSR1	Neurotensin Receptor 1	QT00163373	104
NTSR2	Neurotensin Receptor 1	QT00165151	81
OPN3	Opsin 3	QT00120218	112
OPN5	Opsin 5	QT00140406	101
OPRD1	Opioid Receptor Delta 1	QT00103250	147
OPRK1	Opioid Receptor Kappa 1	QT00101549	73
OPRL1	Opiate Receptor-Like 1	QT00300811	114
OPRM1	Opioid Receptor Mu 1	QT01770811	119
OXGR1	Oxoglutarate (Alpha-Ketoglutarate) Receptor 1	QT01160943	78
OXTR	Oxytocin Receptor	QT01778427	75
P2RY1	Purinergic Receptor P2Y1	QT00158340	96
P2RY10	Purinergic Receptor P2Y, G-Protein Coupled 10	QT00494732	300
P2RY12	Purinergic Receptor P2Y, G-Protein Coupled 12	QT02527000	125

Table A2.2. Continued.

Gene symbol	Gene name	Primer Qiagen Catalogue Number	Amplicon length (bp)
P2RY13	Purinergic Receptor P2Y, G-Protein Coupled 13	QT00124285	73
P2RY14	Purinergic Receptor P2Y, G-Protein Coupled 14	QT00173264	149
P2RY2	Purinergic Receptor P2Y, G-Protein Coupled 2	QT00097202	102
P2RY4	Purinergic Receptor P2Y, G-Protein Coupled 4	QT02332764	107
P2RY6	Purinergic Receptor P2Y, G-Protein Coupled 6	QT00150010	63
PPYR1	Pancreatic Polypeptide Receptor 1	QT00128121	122
PRLHR	Prolactin Releasing Hormone Receptor	QT00326172	60
PROKR1	Prokineticin Receptor 1	QT00314846	91
PROKR2	Prokineticin Receptor 2	QT00157654	78
PTAFR	Platelet-Activating Factor Receptor	QT00262990	89
PTGDR	Prostaglandin D2 Receptor	QT00114310	69
PTGER1	Prostaglandin E Receptor 1	QT00173936	134
PTGER2	Prostaglandin E Receptor 2	QT00115276	68
PTGER3	Prostaglandin E Receptor 3	QT00254303	116
PTGER4	Prostaglandin E Receptor 4	QT00248948	93
PTGFR	Prostaglandin F Receptor	QT00170562	150
PTGIR	Prostaglandin I2 (Prostacyclin) Receptor	QT00160062	120
PTH1R	Parathyroid Hormone 1 Receptor	QT01057784	107
PTH2R	Parathyroid Hormone 2 Receptor	QT00164647	89
QRFPR	Pyroglutamylated RFamide Peptide Receptor	QT02331175	106
RAMP1	Receptor (G Protein-Coupled) Activity Modifying Protein 1	QT02279109	150
RAMP2	Receptor (G Protein-Coupled) Activity Modifying Protein 2	QT00161056	150
RAMP3	Receptor (G Protein-Coupled) Activity Modifying Protein 3	QT00118657	74
RXFP1	Relaxin/Insulin-Like Family Peptide Receptor 1	QT00172382	89
RXFP2	Relaxin/Insulin-Like Family Peptide Receptor 2	QT00131124	80
RXFP3	Relaxin/Insulin-Like Family Peptide Receptor 3	QT00285222	103
RXFP4	Relaxin/Insulin-Like Family Peptide Receptor 4	QT01040375	146
S1PR1	Sphingosine-1-Phosphate Receptor 1	QT00243628	129
S1PR2	Sphingosine-1-Phosphate Receptor 2	QT00262773	87
S1PR3	Sphingosine-1-Phosphate Receptor 3	QT00132160	154
S1PR4	Sphingosine-1-Phosphate Receptor 4	QT00260141	98
S1PR5	Sphingosine-1-Phosphate Receptor 5	QT00282744	134
SCTR	Secretin Receptor	QT01066646	72
SMO	Smoothened Frizzled Class Receptor	QT00494683	104
SSTR1	Somatostatin Receptor 1	QT01761137	98
SSTR2	Somatostatin Receptor 2	QT01539111	150

Table A2.2. Continued.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
SSTR3	Somatostatin Receptor 3	QT01162637	97
SSTR4	Somatostatin Receptor 4	QT02264661	128
SSTR5	Somatostatin Receptor 5	QT01162630	124
SUCNR1	Succinate Receptor 1	QT00127582	118
TAAR1	Trace Amine Associated Receptor 1	QT01041936	93
TAAR2	Trace Amine Associated Receptor 2	QT01066968	140
TAAR5	Trace Amine Associated Receptor 5	QT01064007	127
TAAR6	Trace Amine Associated Receptor 6	QT00360990	133
TAAR8A	Trace Amine Associated Receptor 8A	QT00361382	88
TAAR8B	Trace Amine Associated Receptor 8B	QT00354837	88
TAAR8C	Trace Amine Associated Receptor 8C	QT00331485	88
TAAR9	Trace Amine Associated Receptor 9	QT01071490	97
TACR1	Tachykinin Receptor 1	QT00103334	110
TACR2	Tachykinin Receptor 2	QT00493199	120
TACR3	Tachykinin Receptor 3	QT00134036	109
TAS1R1	Taste Receptor, Type 1, Member 1	QT00121870	92
TAS1R3	Taste Receptor, Type 1, Member 3	QT00309890	110
TAS2R31	Taste Receptor, Type 2, Member 31	QT00327621	68
TBXA2R	Thromboxane A2 Receptor	QT01160873	92
TRHR	Thyrotropin-Releasing Hormone Receptor	QT00161931	108
TSHR	Thyroid Stimulating Hormone Receptor	QT00136955	63
UTS2R	Urotensin 2 Receptor	QT01040263	112
VIPR1	Vasoactive Intestinal Peptide Receptor 1	QT00167160	149
VIPR2	Vasoactive Intestinal Peptide Receptor 2	QT00106463	97
XCR1	Chemokine (C Motif) Receptor 1	QT00261310	97

Table A2.3. List of house-keeping and control gene primers.

Gene symbol	Gene name	Primer Assay Qiagen Catalogue Number	Amplicon length (bp)
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase	QT01658692	144
PRL1	Prolactin	QT00102977	92
PRL3B1	Placental Lactogen-II	QT00123060	64
PRL3D1	Placental Lactogen-I	QT01052219	133
PRLR	Prolactin Receptor	QT00154154	81

Appendix III

Table A3.1 Quantifiable GPCR gene expression in mouse pancreatic islets on gestational day 12, compared to non-pregnant and gestational day 18 expression levels. Trace – detectable but not quantifiable gene expression, Not detectable – not detectable gene expression, n = 3-8.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
ADCYAP1R1	2.45E-03	2.82E-03	6.28E-03
ADORA1	2.01E-02	9.03E-03	3.35E-02
ADORA2A	4.48E-03	7.14E-03	6.09E-03
ADORA2B	7.58E-03	6.56E-03	1.18E-02
ADORA3	2.20E-02	9.15E-03	1.60E-02
ADRA1A	6.65E-04	3.66E-03	3.34E-03
ADRA1D	7.90E-04	5.64E-04	Not detectable
ADRA2A	6.71E-02	5.22E-02	2.02E-01
ADRA2C	3.33E-04	1.18E-03	Not detectable
ADRB1	7.74E-03	2.57E-03	8.54E-03
ADRB2	6.94E-03	3.81E-03	1.78E-02
AGTR1	5.05E-03	6.31E-03	9.42E-03
APLNR	6.94E-03	3.39E-03	3.16E-03
AVPR1A	5.83E-04	6.20E-04	1.28E-02
AVPR1B	2.47E-03	4.38E-03	1.27E-03
AVPR2	Not detectable	5.49E-04	Not detectable
BAI1	7.97E-04	9.27E-04	Not detectable
BAI2	1.09E-03	1.10E-03	8.74E-04
BAI3	2.58E-02	1.47E-02	2.15E-02
BDKRB1	3.05E-04	8.85E-05	2.36E-03
BDKRB2	6.50E-03	5.81E-03	3.00E-02
BRS3	5.03E-04	6.51E-04	Trace
C3AR1	8.00E-03	1.05E-02	5.47E-03
C5AR1	4.82E-04	6.49E-04	5.51E-04
CALCRL	2.25E-02	1.70E-02	3.16E-02
CASR	6.88E-02	4.55E-02	5.06E-02
CCKAR	3.42E-01	2.46E-02	7.93E-04
CCKBR	1.14E-03	4.75E-04	Not detectable
CCR1	7.34E-04	6.05E-04	1.31E-03
CCR10	2.32E-03	1.59E-03	2.50E-03
CCR2	1.03E-03	9.65E-04	3.82E-03

Table A3.1. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
CCR3	2.37E-03	3.05E-04	Trace
CCR4	9.97E-04	7.27E-04	6.09E-04
CCR5	1.62E-03	6.34E-04	1.88E-03
CCR6	7.33E-04	5.51E-04	1.25E-02
CCRL2	1.70E-02	8.27E-03	1.58E-02
CD97	1.44E-02	1.02E-02	2.59E-02
CELSR1	9.50E-02	6.30E-03	1.37E-02
CELSR2	9.98E-03	1.08E-02	1.88E-02
CELSR3	3.95E-02	9.36E-02	1.40E-01
CHRM1	1.03E-03	1.27E-03	7.68E-04
CHRM2	4.33E-03	3.79E-03	1.17E-02
CHRM3	3.76E-02	4.31E-02	8.69E-02
CHRM4	5.56E-03	1.36E-02	8.74E-03
CMKLR1	2.86E-03	2.62E-03	3.64E-03
CRHR1	7.08E-03	5.98E-03	1.49E-03
CRHR2	1.84E-03	2.42E-03	1.25E-03
CX3CR1	4.08E-03	5.46E-03	1.15E-02
CXCR3	Not detectable	3.92E-04	Trace
CXCR4	1.38E-02	7.66E-03	1.91E-02
CXCR5	2.15E-03	1.03E-03	Trace
CXCR7	1.25E-02	1.84E-02	9.39E-03
CYSLTR1	1.71E-03	1.01E-03	2.12E-03
DARC	1.19E-03	7.38E-03	1.56E-03
Drd1a	1.33E-03	5.22E-04	Trace
DRD2	1.20E-03	1.64E-03	1.74E-03
DRD3	2.58E-04	3.16E-04	3.68E-04
EDNRA	1.79E-02	1.56E-02	2.06E-02
EDNRB	1.37E-02	1.05E-02	4.43E-02
ELTD1	3.96E-02	3.34E-02	5.64E-02
EMR1	3.04E-03	2.49E-03	6.78E-03
F2R	1.33E-02	1.54E-02	1.56E-02
F2RL2	7.92E-04	1.26E-03	4.28E-04
F2RL3	2.50E-03	9.54E-04	2.73E-03
FFAR1	2.58E-01	2.04E-01	2.21E-01
FFAR2	1.15E-02	5.25E-03	1.62E-02

Table A3.1. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
FFAR3	3.45E-02	1.79E-02	4.86E-02
FZD1	4.14E-03	1.88E-03	3.17E-03
FZD3	7.89E-02	4.73E-02	7.03E-02
FZD4	1.18E-02	2.73E-03	2.22E-02
FZD6	8.62E-03	4.42E-03	6.86E-03
FZD7	1.07E-02	2.87E-03	2.68E-02
FZD8	1.45E-02	1.52E-02	1.72E-02
FZD9	1.42E-03	3.43E-04	3.36E-04
GABBR1	4.51E-02	1.91E-02	1.42E-01
GABBR2	6.02E-02	9.78E-02	3.25E-01
GALR1	9.72E-02	1.14E-01	9.98E-02
GALR2	2.88E-03	1.72E-03	6.56E-03
GALR3	1.14E-01	1.00E-01	2.39E-01
GCGR	3.87E-02	3.33E-02	7.32E-02
GIPR	8.33E-02	1.46E-01	5.14E-01
GPBAR1	1.36E-03	9.63E-04	9.86E-04
GPER	1.76E-02	3.99E-03	4.49E-03
GPR112	Trace	2.26E-04	Not detectable
GPR116	8.04E-02	2.76E-02	5.14E-02
GPR119	1.33E-02	2.30E-02	7.08E-03
GPR12	Not detectable	1.14E-03	Not detectable
GPR120	1.45E-02	2.60E-02	8.38E-03
GPR123	2.16E-03	1.34E-03	1.92E-03
GPR124	3.21E-02	9.82E-03	4.24E-03
GPR125	3.73E-02	7.35E-03	1.51E-02
GPR126	9.53E-03	2.75E-03	2.07E-03
GPR132	2.59E-03	1.45E-03	1.57E-03
GPR133	5.05E-03	1.05E-03	1.57E-03
GPR135	5.60E-03	6.71E-03	2.59E-03
GPR139	9.76E-04	7.93E-04	Not detectable
GPR141	Not detectable	1.10E-04	Not detectable
GPR142	3.83E-02	2.83E-02	2.63E-02
GPR15	7.81E-04	1.91E-04	2.96E-04
GPR150	2.56E-03	7.36E-04	7.21E-04
GPR151	5.51E-03	7.33E-04	6.92E-03

Table A3.1. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
GPR152	1.09E-03	4.33E-04	Not detectable
GPR153	1.15E-02	5.47E-03	8.89E-03
GPR157	1.03E-02	1.76E-02	2.06E-02
GPR158	1.01E-01	2.08E-01	1.78E-01
GPR160	1.16E-03	1.31E-03	4.39E-04
GPR161	2.91E-02	2.24E-02	2.02E-02
GPR162	Not detectable	2.58E-03	5.68E-03
GPR17	Trace	7.27E-05	Trace
GPR171	1.80E-02	6.10E-04	7.45E-03
GPR173	Not detectable	3.50E-04	Not detectable
GPR179	3.04E-03	1.53E-03	2.33E-03
GPR18	1.06E-02	5.74E-04	6.03E-04
GPR182	1.78E-02	1.34E-03	1.82E-03
GPR183	5.21E-02	1.15E-03	4.21E-03
GPR19	8.17E-02	1.15E-02	2.85E-02
GPR20	7.05E-03	4.87E-04	4.52E-03
GPR25	5.35E-03	1.13E-03	Not detectable
GPR26	4.71E-04	1.68E-03	7.53E-03
GPR27	8.96E-02	6.37E-02	5.40E-02
GPR3	4.38E-03	4.36E-04	6.09E-04
GPR34	4.92E-03	4.61E-04	9.88E-04
GPR37L1	1.61E-03	5.50E-04	6.28E-04
GPR39	2.43E-01	1.55E-02	4.33E-02
GPR4	1.31E-01	7.29E-03	3.50E-02
GPR52	1.72E-02	1.27E-03	4.73E-03
GPR56	6.03E-01	6.19E-01	5.14E-01
GPR62	1.07E-02	4.69E-03	9.82E-03
GPR65	Trace	3.05E-03	1.32E-02
GPR77	Trace	1.48E-03	7.52E-04
GPR84	3.54E-03	1.27E-03	Trace
GPR85	3.59E-02	9.24E-03	2.88E-02
GPR87	1.35E-02	6.65E-04	6.50E-03
GPR88	Not detectable	1.24E-04	Not detectable
GPR98	6.21E-02	1.24E-03	2.58E-02
GPRC5A	6.17E-03	3.19E-03	3.72E-03

Table A3.1. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
GPRC5C	1.18E-01	1.59E-02	8.85E-02
GPRC6A	8.35E-03	3.05E-03	2.08E-03
GRM7	4.54E-03	7.32E-04	Not detectable
HRH3	2.91E-03	5.97E-04	Trace
HTR1B	3.44E-03	6.48E-04	1.64E-03
HTR2B	Not detectable	2.97E-04	Not detectable
KISS1R	8.20E-03	1.67E-03	7.71E-03
LGR4	7.42E-02	4.33E-02	3.52E-02
LPAR3	2.13E-03	7.20E-04	Not detectable
LPAR4	3.21E-04	2.05E-04	5.33E-04
LPAR5	3.17E-03	9.81E-04	4.84E-04
LPAR6	7.99E-02	1.56E-02	5.41E-02
LPHN1	5.08E-01	9.20E-02	2.49E-01
LPHN2	2.15E-02	5.90E-03	1.46E-02
MC1R	1.10E-03	8.06E-04	Not detectable
MC4R	2.59E-03	2.84E-03	2.07E-03
MRGPRE	6.51E-02	2.81E-02	3.06E-02
MRGPRF	4.87E-03	6.54E-03	Not detectable
MRGPRG	6.57E-04	1.44E-04	6.79E-04
NMBR	Not detectable	1.09E-03	4.91E-04
NPFFR2	Trace	1.14E-03	9.07E-04
NPY1R	1.31E-03	1.79E-04	1.82E-03
OPN3	7.65E-03	6.44E-03	2.54E-03
OPRL1	1.59E-02	2.80E-03	1.89E-03
OXTR	5.56E-02	3.35E-02	2.09E-02
P2RY1	2.02E-02	6.57E-03	9.87E-03
P2RY12	1.28E-02	2.40E-03	2.28E-03
P2RY13	2.59E-03	1.33E-03	9.19E-04
P2RY14	5.53E-03	4.23E-03	Not detectable
P2RY2	6.78E-03	1.50E-03	3.69E-03
P2RY4	2.16E-03	1.71E-03	Trace
P2RY6	3.10E-03	1.63E-03	2.45E-03
PTGER1	Not detectable	5.95E-04	Not detectable
PTGIR	2.87E-03	9.57E-04	1.73E-03
RAMP1	2.04E-02	1.66E-02	1.48E-02

Table A3.1. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
RAMP2	Not detectable	5.33E-03	4.99E-02
RAMP3	2.93E-03	5.29E-03	2.50E-02
S1PR1	5.36E-02	3.73E-02	4.67E-02
S1PR2	2.49E-03	3.19E-04	1.14E-03
S1PR4	Not detectable	9.10E-04	1.85E-03
SCTR	2.32E-03	6.43E-04	1.84E-03
SMO	1.48E-02	3.81E-03	6.08E-03
SSTR1	Trace	6.26E-04	1.19E-03
SSTR2	1.31E-02	7.77E-03	4.39E-03
SSTR4	2.19E-03	1.85E-04	8.95E-03
SUCNR1	Trace	8.85E-04	Not detectable
TAAR1	8.27E-02	7.71E-03	1.14E-02
TACR1	No expr	7.85E-04	Not detectable
TAS1R3	5.17E-02	7.25E-03	1.51E-02
TBXA2R	4.47E-03	4.51E-04	1.01E-03
VIPR1	1.75E-01	2.92E-02	5.09E-02
VIPR2	2.12E-03	2.56E-03	2.72E-03
XCR1	6.28E-03	1.46E-03	6.47E-04

Table A3.2. Quantifiable GPCR gene expression in mouse pancreatic islets on gestational day 18, compared to non-pregnant and gestational day 12 expression levels. Trace – detectable but not quantifiable gene expression, Not detectable – not detectable gene expression, n = 3-8.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
ADCYAP1R1	2.45E-03	2.82E-03	6.28E-03
ADORA1	2.01E-02	9.03E-03	3.35E-02
ADORA2A	4.48E-03	7.14E-03	6.09E-03
ADORA2B	7.58E-03	6.56E-03	1.18E-02
ADORA3	2.20E-02	9.15E-03	1.60E-02
ADRA1A	6.65E-04	3.66E-03	3.34E-03
ADRA2A	6.71E-02	5.22E-02	2.02E-01
ADRA2B	1.95E-03	Trace	1.31E-03
ADRB1	7.74E-03	2.57E-03	8.54E-03
ADRB2	6.94E-03	3.81E-03	1.78E-02
AGTR1	5.05E-03	6.31E-03	9.42E-03
APLNR	6.94E-03	3.39E-03	3.16E-03
AVPR1A	5.83E-04	6.20E-04	1.28E-02
AVPR1B	2.47E-03	4.38E-03	1.27E-03
BAI2	1.09E-03	1.10E-03	8.74E-04
BAI3	2.58E-02	1.47E-02	2.15E-02
BDKRB1	3.05E-04	8.85E-05	2.36E-03
BDKRB2	6.50E-03	5.81E-03	3.00E-02
C3AR1	8.00E-03	1.05E-02	5.47E-03
C5AR1	4.82E-04	6.49E-04	5.51E-04
CALCRL	2.25E-02	1.70E-02	3.16E-02
CASR	6.88E-02	4.55E-02	5.06E-02
CCBP2	Trace	Trace	2.69E-03
CCKAR	3.42E-01	2.46E-02	7.93E-04
CCR1	7.34E-04	6.05E-04	1.31E-03
CCR10	2.32E-03	1.59E-03	2.50E-03
CCR2	1.03E-03	9.65E-04	3.82E-03
CCR4	9.97E-04	7.27E-04	6.09E-04
CCR5	1.62E-03	6.34E-04	1.88E-03
CCR6	7.33E-04	5.51E-04	1.25E-02
CCRL2	1.70E-02	8.27E-03	1.58E-02
CD97	1.44E-02	1.02E-02	2.59E-02
CELSR1	9.50E-02	6.30E-03	1.37E-02

Table A3.2. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
CELSR2	9.98E-03	1.08E-02	1.88E-02
CELSR3	3.95E-02	9.36E-02	1.40E-01
CHRM1	1.03E-03	1.27E-03	7.68E-04
CHRM2	4.33E-03	3.79E-03	1.17E-02
CHRM3	3.76E-02	4.31E-02	8.69E-02
CHRM4	5.56E-03	1.36E-02	8.74E-03
CMKLR1	2.86E-03	2.62E-03	3.64E-03
CNR1	5.40E-03	Not detectable	5.19E-03
CNR2	Not detectable	Trace	1.67E-03
CRHR1	7.08E-03	5.98E-03	1.49E-03
CRHR2	1.84E-03	2.42E-03	1.25E-03
CX3CR1	4.08E-03	5.46E-03	1.15E-02
CXCR4	1.38E-02	7.66E-03	1.91E-02
CXCR6	3.48E-04	Not detectable	4.75E-03
CXCR7	1.25E-02	1.84E-02	9.39E-03
CYSLTR1	1.71E-03	1.01E-03	2.12E-03
DARC	1.19E-03	7.38E-03	1.56E-03
DRD2	1.20E-03	1.64E-03	1.74E-03
DRD3	2.58E-04	3.16E-04	3.68E-04
EDNRA	1.79E-02	1.56E-02	2.06E-02
EDNRB	1.37E-02	1.05E-02	4.43E-02
ELTD1	3.96E-02	3.34E-02	5.64E-02
EMR1	3.04E-03	2.49E-03	6.78E-03
F2R	1.33E-02	1.54E-02	1.56E-02
F2RL1	Trace	Not detectable	2.68E-03
F2RL2	7.92E-04	1.26E-03	4.28E-04
F2RL3	2.50E-03	9.54E-04	2.73E-03
FFAR1	2.58E-01	2.04E-01	2.21E-01
FFAR2	1.15E-02	5.25E-03	1.62E-02
FFAR3	3.45E-02	1.79E-02	4.86E-02
FZD1	4.14E-03	1.88E-03	3.17E-03
FZD10	1.04E-03	Not detectable	2.30E-04
FZD2	3.33E-03	Trace	1.15E-03
FZD3	7.89E-02	4.73E-02	7.03E-02
FZD4	1.18E-02	2.73E-03	2.22E-02

Table A3.2. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
FZD5	4.84E-04	Not detectable	2.88E-04
FZD6	8.62E-03	4.42E-03	6.86E-03
FZD7	1.07E-02	2.87E-03	2.68E-02
FZD8	1.45E-02	1.52E-02	1.72E-02
FZD9	1.42E-03	3.43E-04	3.36E-04
GABBR1	4.51E-02	1.91E-02	1.42E-01
GABBR2	6.02E-02	9.78E-02	3.25E-01
GALR1	9.72E-02	1.14E-01	9.98E-02
GALR2	2.88E-03	1.72E-03	6.56E-03
GALR3	1.14E-01	1.00E-01	2.39E-01
GCGR	3.87E-02	3.33E-02	7.32E-02
GHSR	1.02E-02	Not detectable	3.73E-03
GIPR	8.33E-02	1.46E-01	5.14E-01
GPBAR1	1.36E-03	9.63E-04	9.86E-04
GPER	1.76E-02	3.99E-03	4.49E-03
GPR110	1.20E-03	Trace	3.91E-04
GPR116	8.04E-02	2.76E-02	5.14E-02
GPR119	1.33E-02	2.30E-02	7.08E-03
GPR120	1.45E-02	2.60E-02	8.38E-03
GPR123	2.16E-03	1.34E-03	1.92E-03
GPR124	3.21E-02	9.82E-03	4.24E-03
GPR125	3.73E-02	7.35E-03	1.51E-02
GPR126	9.53E-03	2.75E-03	2.07E-03
GPR132	2.59E-03	1.45E-03	1.57E-03
GPR133	5.05E-03	1.05E-03	1.57E-03
GPR135	5.60E-03	6.71E-03	2.59E-03
GPR137	3.14E-03	Not detectable	2.48E-03
GPR142	3.83E-02	2.83E-02	2.63E-02
GPR146	1.49E-02	Not detectable	1.56E-02
GPR15	7.81E-04	1.91E-04	2.96E-04
GPR150	2.56E-03	7.36E-04	7.21E-04
GPR151	5.51E-03	7.33E-04	6.92E-03
GPR153	1.15E-02	5.47E-03	8.89E-03
GPR157	1.03E-02	1.76E-02	2.06E-02
GPR158	1.01E-01	2.08E-01	1.78E-01

Table A3.2. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
GPR160	1.16E-03	1.31E-03	4.39E-04
GPR161	2.91E-02	2.24E-02	2.02E-02
GPR162	Not detectable	2.58E-03	5.68E-03
GPR171	1.80E-02	6.10E-04	7.45E-03
GPR179	3.04E-03	1.53E-03	2.33E-03
GPR18	1.06E-02	5.74E-04	6.03E-04
GPR182	1.78E-02	1.34E-03	1.82E-03
GPR183	5.21E-02	1.15E-03	4.21E-03
GPR19	8.17E-02	1.15E-02	2.85E-02
GPR20	7.05E-03	4.87E-04	4.52E-03
GPR21	8.23E-03	Not detectable	1.17E-03
GPR22	5.92E-03	Not detectable	1.10E-03
GPR26	4.71E-04	1.68E-03	7.53E-03
GPR27	8.96E-02	6.37E-02	5.40E-02
GPR3	4.38E-03	4.36E-04	6.09E-04
GPR34	4.92E-03	4.61E-04	9.88E-04
GPR37L1	1.61E-03	5.50E-04	6.28E-04
GPR39	2.43E-01	1.55E-02	4.33E-02
GPR4	1.31E-01	7.29E-03	3.50E-02
GPR52	1.72E-02	1.27E-03	4.73E-03
GPR56	6.03E-01	6.19E-01	5.14E-01
GPR6	Not detectable	Not detectable	6.82E-05
GPR62	1.07E-02	4.69E-03	9.82E-03
GPR65	Trace	3.05E-03	1.32E-02
GPR77	Trace	1.48E-03	7.52E-04
GPR85	3.59E-02	9.24E-03	2.88E-02
GPR87	1.35E-02	6.65E-04	6.50E-03
GPR97	1.29E-03	Not detectable	3.75E-03
GPR98	6.21E-02	1.24E-03	2.58E-02
GPRC5A	6.17E-03	3.19E-03	3.72E-03
GPRC5B	3.19E-02	Not detectable	6.96E-03
GPRC5C	1.18E-01	1.59E-02	8.85E-02
GPRC6A	8.35E-03	3.05E-03	2.08E-03
GRM8	Not detectable	Not detectable	4.45E-05
HTR1B	3.44E-03	6.48E-04	1.64E-03
KISS1R	8.20E-03	1.67E-03	7.71E-03

Table A3.2. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
LGR4	7.42E-02	4.33E-02	3.52E-02
LPAR1	8.12E-03	Not detectable	5.81E-03
LPAR2	5.26E-03	Not detectable	1.86E-03
LPAR4	3.21E-04	2.05E-04	5.33E-04
LPAR5	3.17E-03	9.81E-04	4.84E-04
LPAR6	7.99E-02	1.56E-02	5.41E-02
LPHN1	5.08E-01	9.20E-02	2.49E-01
LPHN2	2.15E-02	5.90E-03	1.46E-02
LPHN3	Trace	Trace	2.96E-04
LTB4R2	Not detectable	Trace	3.46E-04
MC4R	2.59E-03	2.84E-03	2.07E-03
MCHR1	4.93E-03	Not detectable	2.28E-03
MRGPRE	6.51E-02	2.81E-02	3.06E-02
MRGPRG	6.57E-04	1.44E-04	6.79E-04
NMBR	Not detectable	1.09E-03	4.91E-04
NPFFR2	Trace	1.14E-03	9.07E-04
NPY1R	1.31E-03	1.79E-04	1.82E-03
OPN3	7.65E-03	6.44E-03	2.54E-03
OPRK1	Trace	Not detectable	7.94E-05
OPRL1	1.59E-02	2.80E-03	1.89E-03
OXTR	5.56E-02	3.35E-02	2.09E-02
P2RY1	2.02E-02	6.57E-03	9.87E-03
P2RY12	1.28E-02	2.40E-03	2.28E-03
P2RY13	2.59E-03	1.33E-03	9.19E-04
P2RY2	6.78E-03	1.50E-03	3.69E-03
P2RY6	3.10E-03	1.63E-03	2.45E-03
PTAFR	1.69E-02	Not detectable	1.68E-03
PTGER3	1.57E-02	Not detectable	8.77E-03
PTGIR	2.87E-03	9.57E-04	1.73E-03
PTH1R	Not detectable	No expression	3.47E-03
QRFPR	Not detectable	Trace	7.75E-05
RAMP1	2.04E-02	1.66E-02	1.48E-02
RAMP2	Not detectable	5.33E-03	4.99E-02
RAMP3	2.93E-03	5.29E-03	2.50E-02
RXFP4	Trace	Not detectable	6.46E-04
S1PR1	5.36E-02	3.73E-02	4.67E-02

Table A3.2. Continued.

Gene	Mean expression in non-pregnant islets relative to GAPDH	Mean expression in gestational day 12 islets relative to GAPDH	Mean expression in gestational day 18 islets relative to GAPDH
S1PR2	2.49E-03	3.19E-04	1.14E-03
S1PR3	Trace	7.36E-03	2.83E-02
S1PR4	Not detectable	9.10E-04	1.85E-03
S1PR5	8.67E-04	Trace	7.08E-04
SCTR	2.32E-03	6.43E-04	1.84E-03
SMO	1.48E-02	3.81E-03	6.08E-03
SSTR1	Trace	6.26E-04	1.19E-03
SSTR2	1.31E-02	7.77E-03	4.39E-03
SSTR4	2.19E-03	1.85E-04	8.95E-03
TAAR1	8.27E-02	7.71E-03	1.14E-02
TAAR8a	4.87E-03	Not detectable	1.14E-03
TACR3	1.15E-03	Not detectable	1.25E-03
TAS1R3	5.17E-02	7.25E-03	1.51E-02
TBXA2R	4.47E-03	4.51E-04	1.01E-03
VIPR1	1.75E-01	2.92E-02	5.09E-02
VIPR2	2.12E-03	2.56E-03	2.72E-03
XCR1	6.28E-03	1.46E-03	6.47E-04

Appendix IV

Table A4.1. Effects of GPCR genes, and their ligands, differentially expressed in pancreatic islets on gestational day 12 and 18 compared to non-pregnant expression levels, on cell proliferation and β-cell function.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
ADORA3	Adenosine	Anti-proliferative in mouse testicle Leydig tumour cells [368].	No data	Pro-proliferative in zebra fish pancreatic β-cells [369].	Upregulation of insulin secretion in T1DM mice [370], inhibition of insulin secretion at 50 uM concentration and stimulation at 500 uM concentration in mouse pancreatic islets [371], upregulation of glucagon secretion in the rat pancreas [372].
ADRA1D	Adrenaline Noradrenaline	Pro-proliferative in human prostate cancer cells [373].	No data	Adrenaline: pro-proliferative in pancreatic ductal adenocarcinoma and colon cancer cells [374, 375].	Adrenaline: inhibition of insulin and somatostatin secretion [376-378], stimulation of glucagon secretion [379].
ADRA2A	Adrenaline Noradrenaline	Anti-proliferative in rodent pancreatic β-cells [380, 381].	Inhibition of insulin secretion in rats [380]. Inhibition of insulin secretion, stimulation of glucagon secretion in the rat perfused pancreas [382].	Adrenaline: pro-proliferative in pancreatic ductal adenocarcinoma and colon cancer cells [374, 375].	Adrenaline: inhibition of insulin and somatostatin secretion [376-378], stimulation of glucagon secretion [379].
ADRA2C	Adrenaline Noradrenaline	Anti-proliferative in immature neuronal cells [383], pro-proliferative in mice and human breast cancer cells [384].	Inhibition of insulin secretion in rat and mouse pancreatic islets [376, 377], inhibition of somatostatin secretion in rat pancreatic islets [378].	Adrenaline: pro-proliferative in pancreatic ductal adenocarcinoma and colon cancer cells [374, 375].	Adrenaline: inhibition of insulin and somatostatin secretion [376-378], stimulation of glucagon secretion [379]
ADRB1	Adrenaline Noradrenaline	Anti-proliferative in mouse myoblasts [385].	No data	Adrenaline: pro-proliferative in pancreatic ductal adenocarcinoma and colon cancer cells [374, 375].	Adrenaline: inhibition of insulin and somatostatin secretion [376-378], stimulation of glucagon secretion [379].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
AVPR1A	Arginine vasopressin (AVP) Oxytocin	No data	No data	AVP: pro-proliferative in hamster In-R1-G9 α-cells [245].	AVP: stimulation of insulin and glucagon secretion in mice [244], stimulation of glucagon secretion in In-R1-G9 cells [245].
AVPR1B	Arginine vasopressin (AVP) Oxytocin	Pro-proliferative in hamster In-R1-G9 α-cells [245].	Stimulation of glucagon secretion in In-R1-G9 α-cells [245]. Stimulation of insulin and glucagon secretion in mice [244].	AVP: pro-proliferative in hamster In-R1-G9 α-cells [245].	AVP: stimulation of insulin and glucagon secretion in mice [244], stimulation of glucagon secretion in In-R1-G9 α-cells [245].
AVPR2	Arginine vasopressin (AVP) Oxytocin	No data	No data	AVP: pro-proliferative in hamster In-R1-G9 α-cells [245].	AVP: stimulation of insulin and glucagon secretion in mice [244], stimulation of glucagon secretion in In-R1-G9 cells [245].
BAI3	Orphan	No data	No data	No data	No data
BDKRB1	Kallidin Bradykinin	Pro-proliferative in rat cardiac fibroblasts [386].	No data	Bradykinin: anti-proliferative in mouse glial cells [387], pro-proliferative in human nasal mucosa fibroblasts [388].	Bradykinin: stimulation of insulin and glucagon secretion in the perfused rat pancreas [389, 390], inhibition of somatostatin secretion in the perfused rat pancreas [390].
CCBP2	CCL19 CCL21 CCL25	A decoy receptor, non-signalling	A decoy receptor, non-signalling	No data	No data
CCKAR	Cholecystokinin-4 (CCK-4) CCK-8 CCK-33 Gastrin-17	No data	Stimulation of insulin secretion in rat pancreatic islets [391].	CCK: stimulation of β-cell proliferation in mouse and human pancreatic islets [294].	CCK-8: stimulation of insulin secretion in rat pancreatic islets [391].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
CCKBR	Cholecystokinin-4 (CCK-4) CCK-8 CCK-33 Gastrin-17	Pro-proliferative in human gastric epithelial cells [392].	Upregulation of insulin secretion in mice [293]. Upregulation of somatostatin secretion in rat pancreatic RIN-14B Δ-cells [292].	CCK: stimulation of β-cell proliferation in mouse and human pancreatic islets [294].	CCK-8: stimulation of insulin secretion in rat islets [391].
CCRL2	CCL19 Chemerin	Pro-proliferative in human breast cancer and colorectal cancer cells [393, 394].	No data	CCL19: pro-proliferative in human breast cancer cells [394]. Chemerin: pro-proliferative in rat vascular smooth muscle cells [395].	Chemerin: suppresses insulin secretion in diabetic mice [396].
CELSR1	Orphan	No data	No data	No data	No data
CHRM2	Acetylcholine	No data	No data	Pro-proliferative in pancreatic cancer cells [397].	Stimulation through CHRM1 and CHRM3 receptors, or suppression through CHRM2 and CHRM4 receptors, of insulin secretion in mouse and rat pancreatic islets [398, 399]. Upregulation of insulin secretion through CHRM3 and CHRM5 receptors in human pancreatic islets [400]. Stimulation of somatostatin secretion via CHRM1 receptor in human pancreatic islets [400].
CHRM3	Acetylcholine	Pro-proliferative in mouse prostate cancer cells [401].	No data	Pro-proliferative in pancreatic cancer cells [397].	Stimulation, through CHRM1 and CHRM3 receptors, or suppression, through CHRM2 and CHRM4 receptors, of insulin secretion in mouse and rat pancreatic islets [398, 399]. Upregulation of insulin secretion through CHRM3 and CHRM5 receptors in human pancreatic islets [400]. Stimulation of somatostatin secretion via CHRM1 receptor in human pancreatic islets [400].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
CHRM4	Acetylcholine	No data	Inhibition of insulin secretion in murine and rat pancreatic islets [398, 399].	Pro-proliferative in pancreatic cancer cells [397].	Stimulation, through CHRM1 and CHRM3 receptors, or suppression, through CHRM2 and CHRM4 receptors, of insulin secretion in mouse and rat pancreatic islets [398, 399]. Upregulation of insulin secretion through CHRM3 and CHRM5 receptors in human pancreatic islets [400]. Stimulation of somatostatin secretion via CHRM1 receptor in human pancreatic islets [400].
CRHR1	Corticotrophin-releasing hormone (CRH) Urocortin 1	No data	No data	CRH: pro-proliferative and anti-apoptotic in rat INS-1 β-cells [239]. Urocortin 1: anti-proliferative in human endothelial cells [402].	CRH: stimulation of insulin secretion in rat and mouse pancreatic islets as well as rat INS-1 β-cells [239, 241]. Stimulation of glucagon secretion in rat islets [240].
CX3CR1	CX3CL1	Anti-proliferative in mouse macrophages [332]. Pro-proliferative in human gastric cancer cells [333].	Stimulation of insulin secretion in mice [403].	Anti-apoptotic in human pancreatic islets [267].	Inhibition of glucagon secretion in human pancreatic islets [267].
CXCR3	CXCL9 CXCL10 CXCL11	Anti-proliferative in mouse pancreatic β-cells [324].	No data	CXCL10: anti-proliferative and pro-apoptotic in mouse pancreatic β-cells [324, 404]. CXCL11: anti-proliferative in human cervical carcinoma, rhabdomyo-sarcoma and glioblastoma cell s [405].	No data

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	**Effect on insulin, glucagon, somatostatin secretion**	**Effect on cell proliferation**	**Effect on insulin, glucagon, somatostatin secretion**
CXCR5	CXCL13	Pro-proliferative in human colon cancer cells [406].	No data	Anti-apoptotic in diabetic mouse pancreatic β-cells [327]	No data
DARC	Chemokines CC and CXC	A non-specific decoy receptor, non-signalling	A non-specific decoy receptor, non-signalling	Check CXCR3, CXCR5, CXCR6, CCR3 and CCR7 ligands	Check CXCR3, CXCR5, CXCR6, CCR3 and CCR7 ligands
FFAR3	Short chain free fatty acids: Acetic acid Pentanoic acid Propanoic acid Isobutyric acid Butyric acid	Anti-proliferative and anti-apoptotic in Chinese hamster ovary (CHO) cells [407].	Inhibition of insulin secretion in mouse islets [408].	Butyric acid: anti-proliferative and pro-apoptotic effect in CHO cells [407].	Acetic acid: stimulation of insulin secretion in mice [409]. Propanoic acid: inhibition of insulin secretion in mice [408].
FZD10	WNT7A WNT7B	Pro-proliferative in human hepatic cells [410].	No data	WNT7A: pro-proliferative in human ovarian cancer cells [411]. WNT7B: pro-proliferative in human pancreatic adenocarcino-ma cells [412].	No data

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
FZD4	WNT2 WNT3A WNT5A WNT7B Norrin	Pro-proliferative in mice vascular endothelial cells [413].	No data	WNT2: pro-proliferative in human intestinal epithelial and colorectal cancer cells [414]. WNT3A: pro-proliferative in human acute lymphoblastic leukaemia cells [415]. WNT5A: pro-proliferative and anti-apoptotic in human keratinocytes [416]. WNT7B: pro-proliferative in human pancreatic adenocarcino-ma cells [412]. Norrin: pro-proliferative in human retina cells [417].	No data
FZD7	WNT3 WNT3A WNT5A WNT7A	Pro-proliferative in human chronic myeloid leukaemia (CML) cells [418].	No data	WNT3A: pro-proliferative in human acute lymphoblastic leukaemia cells [415]. WNT5A: pro-proliferative and anti-apoptotic in human keratinocytes [416]. WNT7A: pro-proliferative in human ovarian cancer cells [411]	No data

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
FZD9	WNT2 WNT7A	No data	No data	WNT2: pro-proliferative in human intestinal epithelial and colorectal cancer cells [414]. WNT7A: pro-proliferative in human ovarian cancer cells [411]	No data
GABBR2	γ-Aminobutyric acid (GABA) (forms a functional complex with GABBR1, does not bind the ligand GABA, mediates coupling to G-proteins)	No data	No data	Anti-proliferative in human non-small cell lung cancers (NSCLC) cells [419].	Stimulation of insulin secretion in human pancreatic islets [420].
GALR2	Galanin Galanin-like peptide	Anti-proliferative and pro-apoptotic in head and neck squamous cell carcinoma (HNSCC) [421].	No data	Galanin: Pro-proliferative in human colorectal cancer (CRC) cells [422].	Galanin: inhibition of insulin secretion in mice [423].
GCGR	Glucagon	No data	Stimulation of somatostatin secretion in the perfused human pancreas [424].	Necessary for β-cell differentiation in developing mouse pancreas [425].	Stimulation of insulin secretion in humans [426]. Stimulation of somatostatin secretion in the perfused human pancreas [424]. Stimulation of glucagon secretion in mouse and rat α-cells [427].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
GHSR	Ghrelin Des-Gln14-ghrelin Met-enkephalin	No data	Inhibition of insulin secretion in rat pancreatic islets [428].	Ghrelin: pro-proliferative in mouse pancreatic β-cells [429], anti-apoptotic in mouse and rat pancreatic β-cells [430].	Ghrelin: suppression of insulin and somatostatin secretion in mice and isolated rat pancreatic islets [431, 432], stimulation of glucagon secretion in isolated mouse and rat pancreatic islets [432], restoration of glucose-dependent insulin secretion in mouse pancreatic β-cells exposed to cytokines [430].
GPER	17β-estradiol (E2)	Anti-apoptotic in human pancreatic islets [433].	Stimulation of insulin secretion and inhibition of somatostatin and glucagon secretion in human pancreatic islets [433].	E2 does not affect mouse pancreatic β-cell proliferation and mass in non-pathological conditions [434]. Anti-apoptotic in human pancreatic islets [433].	Stimulation of insulin secretion in humans and mice [433, 435, 436].
GPR112	Orphan	No data	No data	No data	No data
GPR119	Oleoylethanolamide (OEA) Palmitoylethanolamide (PEA) Lysophosphatidylcholine (LPC)	Pro-proliferative in mouse pancreatic β-cells [437].	Stimulation of insulin secretion in mice [437].	No data	OEA: stimulation of insulin release and improved glucose tolerance in mice [438]. PEA: stimulation of insulin release and improved glucose tolerance in mice [438].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
GPR12	Orphan (proposed ligand: sphingosine 1-phosphate, S1P)	No data	No data	Anti-apoptotic but counteracting insulin-mediated stimulation of mouse pancreatic MIN6 β-cell proliferation [439].	Upregulation of insulin secretion in mouse pancreatic MIN6 β-cells and hamster HIT-T 15 β-cells [440-442]. Inhibition of GLP-1-mediated stimulation of insulin secretion in rat pancreatic islets and INS-1 β-cells [443].
GPR124	Glycosaminoglycans (GAGs) of the extracellular membrane	No data	No data	Desulphation of heparan sulphate proteoglycans (HSPGs) reduces β-cell proliferation and potentiates oxidant-induced apoptosis in rat pancreatic islets [444].	No data
GPR125	Orphan	No data	No data	No data	No data
GPR126	Type IV collagen (ColIV)	No data	No data	No data	Co-culture of rat pancreatic islets with extracellular matrix, containing collagen type I mixed with collagen type III, type IV, and laminin, suppresses islet cell death [445].
GPR141	Orphan	No data	No data	No data	No data
GPR15	Orphan	No data	No data	No data	No data
GPR150	Orphan	No data	No data	No data	No data
GPR151	Orphan	No data	No data	No data	No data
GPR162	Orphan	No data	No data	No data	No data
GPR17	Uridine diphosphate (UDP) UDP-glucose UDP-galactose Leukotriene E Leukotriene D Leukotriene C	No data	No data	UDP-glucose: pro-proliferative in glial progenitor cells [446].	UDP: stimulation of insulin and glucagon secretion in isolated mouse pancreatic islets, acting via P2Y6 receptor [447].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
GPR171	Orphan	No data	No data	No data	The hypothalamic GPR171-BigLEN peptide interaction is believed to be involved in regulating feeding, metabolic responses and appetite [448].
GPR173	Orphan	No data	No data	No data	No data
GPR18	Orphan	No data	No data	No data	No data
GPR182	Orphan	No data	No data	No data	No data
GPR25	Orphan	No data	No data	No data	No data
GPR26	Orphan	No data	No data	No data	No data
GPR27	Orphan	No data	Stimulation of insulin secretion in mouse MIN6 β-cells [449].	No data	No data
GPR3	Orphan (proposed ligand: sphingosine 1-phosphate, S1P)	No data	No data	Anti-apoptotic but counteracting insulin-mediated stimulation of mouse pancreatic MIN6 β-cell proliferation [439].	Upregulation of insulin secretion in mouse pancreatic MIN6 β-cells and hamster HIT-T 15 β-cells [440-442]. Inhibition of GLP-1-mediated stimulation of insulin secretion in rat pancreatic islets and INS-1 β-cells [443].
GPR39	Orphan, Proposed ligand: Zink ions	No data	No data	No data	No data
GPR52	Orphan	No data	No data	No data	No data
GPR6	Orphan (proposed ligand: Sphingosine 1-phosphate, S1P)	No data	No data	Anti-apoptotic but counteracting insulin-mediated stimulation of mouse pancreatic MIN6 β-cell proliferation [439].	Upregulation of insulin secretion in mouse pancreatic MIN6 β-cells and hamster HIT-T 15 β-cells [440-442]. Inhibition of GLP-1-mediated stimulation of insulin secretion in rat pancreatic islets and INS-1 β-cells [443].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
GPR65	Proton sensing	Pro-proliferative in NCI-H460 human non-small cell lung cancer cells [450].	No data	Acidic micro-environment can upregulate metastatic progression and cell apoptosis as well as downregulate cancer cell proliferation [451].	No data
GPR77	C3A C4A C5A	Pro-proliferative in human breast cancer cells [452].	No data	C5A: pro-proliferative in human breast cancer cells [452].	No data
GPR88	Orphan	No data	No data	No data	No data
GPR98	Orphan	No data	No data	No data	No data
GPRC5C	Orphan	No data	No data	No data	No data
GPRC6A	Glycine L-alanine L-arginine L-glutamine L-lysine L-ornithine L-serine Osteocalcin	Pro-proliferative in mouse pancreatic β-cells [453, 454].	Stimulation of insulin secretion in mice [453, 454].	Osteocalcin: pro-proliferative in human pancreatic β-cells [455].	L-arginine: stimulation of insulin secretion in mice [454]. Osteocalcin: stimulation of insulin secretion in mouse and human pancreatic islets [274, 455].
GRM8	L-glutamic acid (Glu) (the main endogenous ligand) L-aspartic acid L-serine-O-phosphate *N*-acetylaspartylglutamate, L-cysteine sulfinic acid	Anti-apoptotic and pro-proliferative in neuronal cells [456].	Inhibition of glucagon secretion in rat pancreatic islets [457].	Glu: pro-apoptotic in rat adrenal PC12 cells [458], pro-proliferative in uterine cells and fibroblasts [459].	Glu: stimulation of insulin and somatostatin secretion in rodent pancreatic islets [460-462]; autocrine stimulation of glucagon secretion in human pancreatic α-cells [463].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
HTR1B	Serotonin (5-hydroxytryptamine)	Anti-proliferative in mouse osteoblasts [464].	No data	Pro-proliferative in mouse β-cells [56].	Stimulation of insulin secretion in mice [58].
HTR2B	Serotonin (5-hydroxytryptamine)	Pro-proliferative in mouse β-cells [56].	Stimulation of insulin secretion in mice and isolated human islets [58, 465].	Pro-proliferative in mouse β-cells [56].	Stimulation of insulin secretion in mice [58].
KISS1R	Kisspeptin-10 Kisspeptin-13 Kisspeptin-14 Kisspeptin-52 Kisspeptin-54	Anti-proliferative in human differentiated thyroid and breast cancer cells [466, 467].	Stimulation of glucose-induced insulin secretion in mouse and human pancreatic islets, no effect on the basal rate of insulin secretion [468].	Kisspeptin-10: anti-proliferative in human breast cancer cells [467].	Kisspeptin-54: stimulation of glucose-induced insulin secretion in mouse and human pancreatic islets, no effect on the basal rate of insulin secretion, no effect on glucagon secretion from mouse pancreatic islets [468]. Kisspeptin-13: suppression of insulin secretion, no effect on glucagon and somatostatin release from the perfused rat pancreas [469].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
LGR4	RSPO1 RSPO2 RSPO3 RSPO4	Pro-proliferative in multiple cancer cell lines [470, 471]. Pro-proliferative in mouse epithelial cells [472].	No data	RSPO1: pro-proliferative and anti-apoptotic in murine pancreatic MIN6 β-cells [162], downregulates pancreatic β-cell neogenesis in mice [163]. RSPO2: pro-proliferative in human keratinocytes [473]. RSPO3: may upregulate human pancreatic β-cell proliferation *in vitro* [335].	RSPO1: stimulation of insulin secretion in mouse pancreatic MIN6 β-cells [163].
LPAR2	Lysophosphatidic acid (LPA)	Pro-proliferative in mouse colorectal cancer cells [474].	No data	Pro-proliferative in murine ovarian cancer cells [475], inhibits adipogenesis [476].	Suppression of insulin secretion in mice [477].
LPHN1	α-latrotoxin	No data	No data	No data	α-latrotoxin exerts GLP-1-mimetic actions and stimulates insulin secretion from human pancreatic β-cells and mouse MIN6 β-cells [478, 479].
LPHN2	α-latrotoxin	No data	No data	No data	α-latrotoxin exerts GLP-1-mimetic actions and stimulates insulin secretion from human pancreatic β-cells and mouse MIN6 β-cells [478, 479].
LPHN3	Fibronectin leucine rich transmembrane protein 3 (FLRT3)	No data	No data	No data	No data

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
LTB4R2	Leukotriene B4 (LTB4) 20-hydroxy-LTB4 12-hydroxyheptadecatrienoic acid (12-HHT) Hydroxyeicosatetraenoic acid (HETE)	Pro-proliferative in human pancreatic and bladder cancer cells [480, 481].	No data	LTB4: pro-proliferative in human lymphocytic leukaemia cells [482].	LTB4: stimulation of insulin secretion in rat pancreatic islets [483].
NMBR	Neuromedin B (NMB) Gastrin releasing peptide (GRP) GRP18-27 (neuromedin C) Bombesin	Pro-proliferative in rat osteoblasts [287].	No data	Neuromedin B: pro-proliferative in rat osteoblasts [287].	Neuromedin B: stimulation of insulin secretion and no effect on glucagon and somatostatin release in the canine pancreas [285], no effect on insulin and a stimulatory effect on glucagon secretion in the rat pancreas [284]. GRP: stimulation of insulin secretion in mice [484]. Bombesin: enhancement of extendin-4-mediated upregulation of insulin secretion[485].
NPFFR2	Neuropeptide AF (A-18-F-amide) Neuropeptide FF (F-8-F-amide) Neuropeptide SF	No data	No data	No data	Neuropeptide AF: inhibition of insulin and somatostatin secretion in the perfused rat pancreas [486]. Neuropeptide FF: inhibition of insulin and somatostatin secretion in the perfused rat pancreas [486].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
OPRK1	Opioids (dynorphin A and dynorphin B are the most potent ligands)	No data	No data	Dynorphin A: anti-proliferative in rat glial cells [487] Dynorphin B: anti-proliferative in rat glial cells [487].	Dynorphin A: inhibition of insulin secretion in the isolated rat pancreas [488], stimulation of insulin secretion and no effect on somatostatin release in rat pancreatic islets [489], stimulation of glucagon secretion in mouse pancreatic islets [490].
OPRL1	Nociceptin (PNOC)	Pro-proliferative in murine spermatocytes [491].	No data	Pro-proliferative in murine spermatocytes [491]. Anti-proliferative in human T cells [492].	No data
OXTR	Oxytocin Arginine vasopressin	Anti-apoptotic in mouse pancreatic β-cells [493].	Stimulation of insulin secretion in mouse pancreatic β-cells [493].	Oxytocin: anti-apoptotic in mouse pancreatic β-cells [493], anti-proliferative in human dermal fibroblasts and keratinocytes [494], pro-proliferative in mouse adipose tissue-derived stem cells (ADSCs) [495]. AVP: pro-proliferative in hamster In-R1-G9 α-cells [245].	Oxytocin: stimulation of insulin, glucagon and somatostatin secretion in mouse islets [496]. AVP: stimulation of insulin and glucagon secretion in mice [244], stimulation of glucagon secretion in In-R1-G9 cells [245].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
P2RY1	Adenosine diphosphate (ADP) Adenosine triphosphate (ATP)	No data	Stimulation of insulin secretion in mouse pancreatic islets [497].	ADP: pro-apoptotic in mouse pancreatic MIN6 β-cells, acting on the P2RY13 receptor [498]. ATP: pro-proliferative in the mouse developing retina [499].	ADP: inhibition of insulin and glucagon secretion in mice through P2Y13 receptor activation [497], stimulation of insulin secretion in mouse pancreatic islets through the P2RY1 receptor [497]. ATP: stimulation of insulin secretion through P2RY11 activation, inhibition of insulin secretion through P2RX7 activation in hamster HIT-T1 β-cells [500]
P2RY12	Adenosine diphosphate (ADP)	No data	No data	Pro-apoptotic in mouse pancreatic MIN6 β-cells, acting via P2RY13 receptor [498].	ADP: inhibition of insulin and glucagon secretion in mice through P2Y13 receptor activation [497], stimulation of insulin secretion in mouse pancreatic islets through the P2RY1 receptor [497].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
P2RY13	Adenosine diphosphate (ADP) Adenosine triphosphate (ATP)	Pro-apoptotic in mouse MIN6c4 β-cells [501].	Inhibition of insulin and glucagon secretion in mouse MIN6c4 β-cells [497].	ADP: pro-apoptotic in mouse pancreatic MIN6 β-cells, acting through the P2RY13 receptor [498]. ATP: pro-proliferative in the mouse developing retina [499].	ADP: inhibition of insulin and glucagon secretion in mice through P2Y13 receptor activation [497], stimulation of insulin secretion in mouse pancreatic islets through the P2RY1 receptor [497]. ATP: stimulation of insulin secretion through P2RY11 activation, inhibition of insulin secretion through P2RX7 activation in hamster HIT-T1 β-cells [500]
PTGER1	Prostaglandin E2 (PGE2) (the principal ligand) Prostaglandin F2α (PGF2α) Prostacyclin (PGI2) Prostaglandin D2 (PGD2) Thromboxane A2 (TBXA2)	Pro-proliferative in human hepatocellular carcinoma [502].	No data	PGE2: pro-proliferative in human hepatocellular carcinoma [502]. PGI2: anti-apoptotic in rat pancreatic RINm5F β-cells [503] and human pulmonary endothelial cells [504]. TBXA2: pro-proliferative in human coronary artery smooth muscle cells [505].	PGE2: inhibition of insulin and glucagon secretion in rodent islets [506, 507], stimulation of somatostatin release from the rat pancreas [508]. PGD2: stimulation of insulin and glucagon secretion, no effect on somatostatin release in the rat pancreas [508, 509]. TBXA2: no effect on Insulin and glucagon secretion in the perfused rat pancreas [506].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
PTGIR	Prostacyclin (PGI2) – the principal ligand Prostaglandin E2 (PGE2), Prostaglandin F2α (PGF2α), Prostaglandin D2 (PGD2), Thromboxane A2 (TBXA2)	Anti-proliferative in rat cardiac fibroblasts [510].	No data	PGE2: pro-proliferative in human hepatocellular carcinoma [502]. PGI2: anti-apoptotic in rat pancreatic RINm5F β-cells and human pulmonary endothelial cells [503, 504]. TBXA2: pro-proliferative in human coronary artery smooth muscle cells [505].	PGE2: inhibition of insulin and glucagon secretion in rodent islets [506, 507], stimulation of somatostatin release from the rat pancreas [508]. PGD2: stimulation of insulin and glucagon secretion, no effect on somatostatin release in the rat pancreas [508, 509]. TBXA2: no effect on insulin and glucagon secretion [506].
PTH1R	Parathyroid hormone (PTH) Parathyroid hormone-related protein (PTHrP) PTHrP-(1-36)	Pro-proliferative in mouse osteosarcoma cells [511].	No data	PTHrP: pro-proliferative in rat and human pancreatic β-cells [512, 513]. PTHrP-(1-36): pro-proliferative in human pancreatic β-cells [512].	PTHrP: stimulation of insulin secretion in rat pancreatic islets [513]. PTHrP-(1-36): stimulation of insulin secretion in human pancreatic islets [512]. PTH: suppression of insulin secretion in rats (chronic administration) [514], upregulation of glucagon secretion in the perfused rat pancreas [515].
QRFPR	Pyroglutamylated RFamide peptide (QRFP43)	No data	No data	Anti-apoptotic in rat pancreatic INS-1E β-cells and human pancreatic islets [516].	Stimulation of insulin secretion in rat pancreatic INS-1E β-cells and human pancreatic islets [516].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
RAMP2	Adrenomedullin (ADM) (the principal ligand) Amylin (a heterocomplex of RAMP2 and the CRLR receptor forms the AM1 receptor)	No data	No data	ADM: pro-proliferative in human colorectal cancer (CRC) cells [308] and osteoblasts [307]. Amylin: pro-proliferative in human osteoblasts [307].	ADM: inhibition of insulin secretion in rat islets [305], no effect on glucagon secretion in rat islets [306].
RAMP3	Adrenomedulin (the principal ligand) Amylin (a heterocomplex of RAMP3 and the CRLR receptor forms the AM2 receptor)	Pro-apoptotic in human thymic lymphoma cells [517].	No data	ADM: pro-proliferative in human colorectal cancer (CRC) cells [308] and osteoblasts [307]. Amylin: pro-proliferative in human osteoblasts [307].	ADM: inhibition of insulin secretion in rat islets [305], no effect on glucagon secretion in rat islets [306].
RXFP4	Insulin-like peptide 5 (INSL5) Relaxin-3	No data	No data	INSL5: pro-proliferative in mouse pancreatic β-cells [518].	INSL5: Stimulation of insulin secretion in mice [518, 519].
S1PR2	Sphingosine 1-phosphate (S1P) Sphingosylphosphoryl-choline	Anti-apoptotic but counteracting insulin-mediated stimulation of mouse pancreatic MIN6 β-cell proliferation [439]. Anti-proliferative in mouse preadipocytes [520].	Inhibition of insulin secretion in mice [520].	S1P: anti-apoptotic but counteracting insulin-mediated stimulation of mouse pancreatic MIN6 β-cell proliferation [439]. Pro-proliferative in mouse preadipocytes [520].	S1P: upregulation of insulin secretion in mouse pancreatic MIN6 β-cells and hamster HIT-T 15 β-cells [440-442], Inhibition of GLP-1-mediated stimulation of insulin secretion in rat pancreatic islets and INS-1 β-cells [443].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
S1PR3	Sphingosine 1-phosphate (S1P) Sphingosylphosphoryl-choline	Pro-proliferative in mouse cancer stem cells [521].	No data	S1P: anti-apoptotic but counteracting insulin-mediated stimulation of mouse pancreatic MIN6 β-cell proliferation [439]. Pro-proliferative in mouse preadipocytes [520].	S1P: upregulation of insulin secretion in mouse pancreatic MIN6 β-cells and hamster HIT-T 15 β-cells [440-442], inhibition of GLP-1-mediated stimulation of insulin secretion in rat pancreatic islets and INS-1 β-cells [443].
S1PR4	Sphingosine 1-phosphate (S1P) Sphingosylphosphorylcho-line	No data	No data	S1P: anti-apoptotic but counteracting insulin-mediated stimulation of mouse pancreatic MIN6 β-cell proliferation [439]. Pro-proliferative in mouse preadipocytes [520].	S1P: upregulation of insulin secretion in mouse pancreatic MIN6 β-cells and hamster HIT-T 15 β-cells [440-442]. Inhibition of GLP-1-mediated stimulation of insulin secretion in rat pancreatic islets and INS-1 β-cells [443].
SCTR	Secretin	Ani-proliferative in normal human breast cells, pro-proliferative in human MCF-7 breast cancer cells [522]. Pro-proliferative in rat adrenal cancer cells [523].	No data	No data	Potentiation of insulin and glucagon secretion in mouse pancreatic islets [524, 525].
SSTR1	Somatostatin-14 (SRIF-14) Somatostatin-28 (SRIF-28) Cortistatin-14 (CST-14) Cortistatin-17 (CST-17)	Pro-apoptotic in rat neuronal cells [526].	Inhibition of insulin and glucagon secretion in human islets [527].	Somatostatin: anti-proliferative in human cells [235]. Cortistatin: anti-proliferative in human keratinocytes [528].	Somatostatin: inhibition of insulin and glucagon secretion in humans [529, 530]. Cortistatin: inhibition of insulin and glucagon secretion in humans [531].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	**Effect on insulin, glucagon, somatostatin secretion**	**Effect on cell proliferation**	**Effect on insulin, glucagon, somatostatin secretion**
SSTR4	Somatostatin-14 (SRIF-14) Somatostatin-28 (SRIF-28) Cortistatin-14 (CST-14) Cortistatin-17 (CST-17	No data	No data	Somatostatin: anti-proliferative in human cells [235]. Cortistatin: anti-proliferative in human keratinocytes [528].	Somatostatin: inhibition of insulin and glucagon secretion in humans [529, 530]. Cortistatin: inhibition of insulin and glucagon secretion in humans [531].
SUCNR1	Succinate	No data	No data	No data	No data
TAAR1	Trace amines: Tyramine (the most potent) 3-iodothyronamine β-phenylethylamine Octopamine Dopamine 3-iodothyronamine	Pro-apoptotic in human lymphoma cells [532].	Stimulation of insulin secretion in rat pancreatic INS-1E β-cells and human islets [533].	Dopamine: anti-proliferative in rat pancreatic β-cells [534].	3-iodothyrona-mine: inhibition of insulin secretion, stimulation of glucagon secretion in mice and rats [107, 535].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
TACR1	Substance P (the most potent ligand) Neurokinin A Neurokinin B	Pro-proliferative in murine chondrocytes [536].	Stimulation of insulin and glucagon secretion in the isolated porcine pancreas [537].	Substance P: pro-proliferative in murine chondrocytes [536]. Neurokinin A: anti-proliferative in human hematopoietic progenitor cells [538].	Substance P: stimulation of insulin and glucagon secretion, no effect on somatostatin secretion in the porcine pancreas [537]; stimulation of insulin, glucagon and somatostatin secretion in the canine pancreas [539]; suppression of insulin and glucagon secretion, no effect on somatostatin secretion in the rat pancreas [539]. Neurokinin A: stimulation of insulin and, glucagon, no effect on somatostatin secretion in the porcine pancreas [537].
TAS1R3	Sweet taste receptor Amino acid receptor when dimerised with TAS1R1	No data	The TAS1R1-TAS1R3 dimer upregulates insulin secretion in mouse pancreatic MIN6 β-cells [540].	Leucine: pro-proliferative in rodent pancreatic β-cells [541].	Leucine: stimulation of insulin secretion in rodent pancreatic islets [541]. L-glutamate: upregulation of insulin secretion in mouse pancreatic MIN6 β-cells via TAS1R1-TAS1R3 receptor [540]. L-arginine: upregulation of insulin secretion in mouse pancreatic MIN6 β-cells via TAS1R1-TAS1R3 receptor [540].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion	Effect on cell proliferation	Effect on insulin, glucagon, somatostatin secretion
TBXA2R	Thromboxane A2 (TBXA2) (the principal ligand) Prostacyclin (PGI2) Prostaglandin E2 (PGE2) Prostaglandin F2α (PGF2α) Prostaglandin D2 (PGD2)	Pro-proliferative in human airway smooth muscle cells [542].	No data	PGE2: pro-proliferative in human hepatocellular carcinoma [502]. PGI2: anti-apoptotic in rat pancreatic RINm5F β-cells and human pulmonary endothelial cells [503, 504]. TBXA2: pro-proliferative in human coronary artery smooth muscle cells [505].	TBXA2: no effect on insulin and glucagon secretion [506]. PGE2: inhibition of insulin and glucagon secretion in rodent islets [506, 507], stimulation of somatostatin release from the rat pancreas [508]. PGD2: stimulation of insulin and glucagon secretion, no effect on somatostatin release in the perfused rat pancreas [508, 509].
VIPR1	Principal ligands: Vasoactive intestinal peptide (VIP) Pituitary adenylate cyclase activating polypeptide-38 (PACAP-38) Pituitary adenylate cyclase activating polypeptide-27 (PACAP-27) Additional ligands: Pre-pro VIP (PHI) Secretin Growth hormone releasing hormone (GHRH)	Pro-proliferative in human lymphoblastoma cells [543].	Stimulation of insulin secretion in mice [544].	PACAP: overexpression in mice leads to an increase in the pancreatic islet size [545]. VIP: anti-proliferative in renal cell carcinoma and rat aortic and pulmonary artery smooth muscle cells [546, 547]. Secretin: pro-proliferative in mouse biliary cells [548].	PACAP: stimulation of insulin secretion in mice and both insulin and glucagon secretion in humans [544, 549]. VIP: stimulation of insulin, glucagon and somatostatin release in rat pancreatic islets [550]. Secretin: stimulation of insulin secretion in mice [551].

Table A4.1. Continued.

Receptor	Ligand	Receptor		Ligand	
		Effect on cell proliferation	**Effect on insulin, glucagon, somatostatin secretion**	**Effect on cell proliferation**	**Effect on insulin, glucagon, somatostatin secretion**
XCR1	Chemokine (C Motif) Ligand 1 (XCL1) Chemokine (C Motif) Ligand 2 (XCL2)	Pro-proliferative in human lung cancer cells [552].	No data	XCL1: pro-proliferative in human lung cancer cells [552]. XCL2: pro-proliferative in human epithelial ovarian carcinoma (EOC) [553].	No data

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